The Examiner stated that applicant is reminded of the proper language and format for an abstract of the disclosure. The Examiner stated that the abstract should be in narrative form and generally limited to a single paragraph on a separate sheet within the range of 50 to 150 words. The Examiner stated that it is important that the abstract not exceed 150 words in length since the space provided for the abstract on the computer tape used by the printer is limited. The Examiner stated that the form and legal phraseology often used in patent claims, such as "means" and "said," should be avoided. The Examiner stated that the abstract should describe the disclosure sufficiently to assist readers in deciding whether there is a need for consulting the full patent text for details. The Examiner stated that the language should be clear and concise and should not repeat information given in the title. The Examiner stated that it should avoid using phrases which can be implied, such as, "The disclosure concerns," "The disclosure defined by this invention," "The disclosure describes," etc. The Examiner stated that the instant abstract contains the term, "said" in line 13. The Examiner stated that appropriate correction is requested.

In response, applicants have hereinabove amended the abstract of the disclosure to recite in-part "which encodes amyloid-beta peptide alcohol dehydrogenase (ABAD), and exhibits at least one phenotype from the group consisting of." Therefore, the abstract of the disclosure no longer recites the alleged limitation "**wherein said non-human animal** exhibits at least one phenotype from the

group consisting of" [emphasis added]. Applicants contend that this amendment obviates the Examiner's above objection. Accordingly, applicants respectfully request that the Examiner reconsider and withdraw this ground of objection.

Disclosure:

The Examiner stated that the disclosure is objected to because of the following alleged informalities:

The Examiner stated that p.5, line 33 states that the transgenic non-human mammals of the invention comprise a nucleic acid sequence encoding RAGE, however, the specification does not discuss RAGE.

In response, applicants maintain that contrary to the Examiner's above statement, page 5, line 33 does not recite a nucleic acid sequence encoding "RAGE". Applicants contend that at page 5, line 33, the specification recites "β-hydroxybutyrate (BHB) levels determined on extracts of whole brains". Accordingly, applicants respectfully request that the Examiner reconsider and withdraw the above objection.

The Examiner stated that p.8, lines 8-9 recite "at least one phenotype from the group consisting of elevated levels of basal ATP." The Examiner alleged that there is no group described, thus the phrase is confusing.

In response, applicants have hereinabove amended the specification

to correct an obvious error at page 8, lines 8-9. The specification now recites "wherein said non-human animal exhibits at least one phenotype from the group consisting of: overexpression of ABAD, elevated levels of basal ATP; protection from metabolic or ischemic stress." In support of such amendment to the specification, applicants respectfully direct the Examiner to the Summary of the Invention which recites as follows:

> "The present invention provides for a transgenic non-human animal whose cells contain a recombinant DNA sequence comprising a nerve tissue specific promoter operatively linked to a DNA sequence which encodes amyloid-beta peptide alcohol dehydrogenase (ABAD), wherein said non-human animal exhibits at least one phenotype from the group consisting of: **overexpression of ABAD, elevated levels of basal ATP; protection from metabolic or ischemic stress**" [Emphasis Added] (See page 3, lines 3-10).

Accordingly, applicants contend that the Summary of the Invention at page 3, lines 3-10, as a summary of the detailed description of the invention, demonstrates that the exclusion of the phenotypes "overexpression of ABAD" and "protection from metabolic or ischemic stress" was an obvious error and such amendment to the specification does not introduce any new matter.

The Examiner alleged that p.15, line 33 discusses a nucleic acid sequence encoding RAGE, yet this is not described further in the specification. The Examiner stated that clarification and/or amendment is requested.

In response, applicants have hereinabove amended the specification to correct an obvious error at page 15, line 33. The specification now recites "ABAD" rather than "RAGE" In support of such an amendment to the specification, applicants respectfully direct the Examiner to the Summary of the Invention which recites as follows:

> "The present invention provides for a transgenic non-human animal whose cells contain a recombinant DNA sequence comprising a nerve tissue specific promoter operatively linked to a DNA sequence which encodes **amyloid-beta peptide alcohol dehydrogenase (ABAD)**, wherein said non-human animal exhibits at least one phenotype from the group consisting of: overexpression of ABAD, elevated levels of basal ATP; protection from metabolic or ischemic stress" [Emphasis Added] (See page 3, lines 3-10).

Accordingly, applicants contend that the Summary of the Invention at page 3, lines 3-10, as a summary of the presently claimed invention, demonstrates that the recitation of "RAGE" was an obvious error and such amendment to the specification does not introduce any new matter.

The Examiner alleged p.17, line 21 recites the term, "excitotoxic stress" and that the disclosure does not define this term.

In response, applicants contend that the term "excitotoxic stress" is a term of art and therefore would be well known to one of skill in the art. In support, applicants attach hereto as **Exhibit C** a copy of a paper by Almli et al. entitled "Multiple pathways of

neuroprotection against oxidative stress and excitotoxic injury in immature primary hippocampal neurons" that recites "it is well documented that excitotoxicity and oxidative stress contribute to the pathogenesis of many neurological disorders." (See page 121, first column). Further, Almli et al. recite that "neuroprotection from different types of toxins suggests multiple pathways between excitotoxic (NMDA) and oxidative (H_2O_2) stress in the immature nervous system." (See page 126, first column). Accordingly, this paper demonstrates that the term "excitotoxic stress" is a term of art that one of skill in the art would understand and does not need to be defined in the specification.

The Examiner stated p.17, line 27 recites the term, "cels." The Examiner stated that clarification and/or amendment is requested.

In response, applicants have hereinabove amended the specification at page 17, line 27, to recite "cells" rather than "cels".

The Examiner alleged that p.32 discusses the transgene construct and refers to figures 1A-B(see lines 25 and 28), yet the submitted figure 1 allegedly does not have parts A and B.

In response, applicants attach herewith as **Exhibit D** an amended figure 1 wherein the schematic representation of the transgene (i.e. PD-huABAD construct) used to make the transgenic mouse of the present invention is now labeled "fig. 1A" and the ~3kb fragment (i.e. PD-huABAD transgenic cassette)containing the promoter, cDNA

and required other sequences excised from the plasmid backbone as a PvuI fragment is now labeled "fig. 1B." Accordingly, applicants contend that the Brief Description of the Figures at page 4, lines 3-5 describe the content of figure 1A and 1B, and as a summary of the content of figure 1 of the presently claimed invention, demonstrate that the omission of "1A" and "1B" from figure 1 was an obvious error and such amendment to the specification does not introduce any new matter.

The Examiner alleged that p.34-35, bridging paragraph discusses the cross breeding Tg PD-RAGE mice with Tg hAPP mice, however double transgenic mice expressing both hAPP and ABAD transgene were identified. The Examiner alleged that it is not clear which transgene these mice are expressing (RAGE or ABAD).

In response, applicants have hereinabove amended the specification to correct an obvious error at page 34, line 29. The specification now recites "PD-ABAD" rather than "PD-RAGE" In support of such an amendment to the specification, applicants respectfully direct the Examiner to the specification at page 35, lines 3-4, which recites that "cross-breeding was performed and double-transgenic **mice expressing both hAPP and PD-ABAD transgenes** were identified with specific primers" [Emphasis added]. Accordingly, applicants contend that the specification at page 35, lines 3-5 demonstrates that the recitation of "PD-RAGE" was an obvious error and such amendment to the specification does not introduce any new matter.

The Examiner alleged that p.41-42, bridging paragraph discusses the induction of stroke in TgPD-RAGE mice, however, the discussion on p.45 allegedly discusses the results of induction of stroke in TgPD-ABAD mice.

In response, applicants have hereinabove amended the specification to correct an obvious error at page 41, lines 15-16. The specification now recites "Induction of stroke in Tg **PD-ABAD** mice. Functional consequences of overexpression of **ABAD** were first assessed" [Emphasis added]. Therefore, the specification no longer recites the alleged limitation "PD-RAGE" or "RAGE". In support of such an amendment to the specification, applicants respectfully direct the Examiner to the specification at page 45, lines 13-35 and page 46, lines 1-5, which recites the results of induction of stroke in **Tg PD-ABAD mice.** Accordingly, applicants contend that the results of induction of stroke in Tg PD-ABAD mice page 45, lines 13-35 and page 46, lines 1-5 at page 45, as a description of the experimental results, demonstrate that the recitation of "Tg PD-RAGE" and "RAGE" was an obvious error and such amendment to the specification does not introduce any new matter.

The Examiner alleged that p. 48, lines 4-9 discusses the characterization of neurons isolated from Tg PD-ABAD mice, yet allegedly states that there are high levels of RAGE expression(in particular, see line 8).

In response, applicants have hereinabove amended the specification

to correct an obvious error at page 48, line 8. The specification now recites that "cultured neurons displayed high levels of **ABAD** expression based on Northern analysis and immunoblotting." Therefore, the specification no longer recites the alleged limitation "RAGE". In support of such an amendment to the specification, applicants respectfully direct the Examiner to the specification at page 48, lines 4-8, which recite the characterization of neurons isolated from **Tg PD-ABAD** mice which expressed high levels of ABAD. Accordingly, applicants contend that the characterization of neurons isolated from Tg PD-ABAD mice as described at page 48, lines 4-9, demonstrate that the recitation of "RAGE" was an obvious error and such amendment to the specification does not introduce any new matter.

The Examiner alleged that p.62, line 23, states, "Neurological Exam:" and then a blank space following.

In response, applicants have hereinabove amended the specification to correct an obvious error at page 62, line 23. The specification no longer contains a blank space following "Neurological Exam:". In support of such an amendment to the specification, applicants respectfully direct the Examiner to the specification at page 42, line 23 which recites the same "Neurological Exam:" as at page 62, line 23 and does not contain the blank space. Accordingly, applicants contend that the blank space following "Neurological Exam:" at page 62, line 23, demonstrates an obvious error and such amendment to the specification does not introduce any new matter.

The Examiner alleged that pp.65-66 discuss ABAD and the response of COS cells to nutritional stress. The Examiner alleged that they refer to Figures 3A, 3B and 3C, however, there is allegedly only one Figure 3.

In response, applicants have hereinabove amended the specification to correct an obvious error at pages 65-66. The specification no longer recites "(Fig. 3A)" at page 65, line 31 and page 66, line 6; "(Fig. 3B)" at page 66, line 15; "(Fig. 3C)" at page 65, line 32 and page 66, line 7; and "(Fig. 3A,C)" at page 66, line 27. In support of such an amendment to the specification, applicants respectfully direct the Examiner to the Brief Description of the Figures at page 4, lines 11-12 which recites "Figure 3. Identification of Tg PD-ABAD mice (+) and nontransgenic littermate controls (-) by PCR." Accordingly, applicants contend that the Brief Description of the Figures at page 4, lines 11-12 describe the content of figure 3, and as a summary of the content of figure 3 of the presently claimed invention, demonstrate that the inclusion of "(Fig. 3A)" at page 65, line 31 and page 66, line 6; "(Fig. 3B)" at page 66, line 15; "(Fig. 3C)" at page 65, line 32 and page 66, line 7; and "(Fig. 3A,C)" at page 66, line 27 was an obvious error and such amendment to the specification does not introduce any new matter.

The Examiner alleged that pp.66-67 discuss NMR studies performed on cells, and refer to Figure 4A and 4B, yet Figure 4 allegedly does not recite any NMR results.

In response, applicants have hereinabove amended the specification to correct an obvious error at pages 66-67. The specification no longer recites "(Fig. 4A)" at page 67, line 6; and "(Fig. 4B; P<0.03 at days 4, 6, 8)" at page 67, line 15. In support of such an amendment to the specification, applicants respectfully direct the Examiner to the Brief Description of the Figures at page 4, lines 14-23 which describes ABAD expression in Tg PD-ABAD mice (+) compared with nontransgenic littermate controls (-). Accordingly, applicants contend that the Brief Description of the Figures at page 4, lines 14-23 describe the content of figures 4A-4D of the presently claimed invention and demonstrate that the inclusion of "(Fig. 4A)" at page 67, line 6; and "(Fig. 4B; P<0.03 at days 4, 6, 8)" at page 67, line 15 was an obvious error and such amendment to the specification does not introduce any new matter

The Examiner alleged that pp.67-68 discuss the upregulation of ABAD in response to cerebral ischemia, referring to Figures 5A, B and C, however, there is allegedly only one Figure 5.

In response, applicants have hereinabove amended the specification to correct an obvious error at page 68. The specification no longer recites "(Fig. 5A)" at page 68, line 3; "(Fig. 5A, inset)" at page 68, line 4; "(Fig. 5B) at page 68, line 5; and (Fig. 5C) at page 68, line 9. In support of such an amendment to the specification, applicants respectfully direct the Examiner to the Brief Description of the Figures at page 4, lines 25-28 which describes RAGE expression in brain subregions of Tg PD-ABAD mice

compared to nontransgenic littermate controls. Accordingly, applicants contend that the Brief Description of the Figures at page 4, lines 25-28 describe the content of figure 5 of the presently claimed invention and demonstrate that the inclusion of "(Fig. 5A)" at page 68, line 3; "(Fig. 5A, inset)" at page 68, line 4; "(Fig. 5B) at page 68, line 5; and (Fig. 5C) at page 68, line 9. was an obvious error and such amendment to the specification does not introduce any new matter

The Examiner alleged that p.68 discusses the characterization of Tg PD-ABAD mice, and refers to Figures 6A, 6B, 6C, 6D1, 6D2, 6D3, yet, for example, Figure 6A which allegedly describes on p.68, line 22 as showing mRNA levels, is an NMR result. The Examiner alleges furthermore, that figures 6D1-3 are not present.

In response, applicants have hereinabove amended the specification to correct an obvious error at pages 68-69. The specification no longer recites "(Fig. 6A)" at page 68, line 22; "(Fig. 6B)" at page 68, line 22; "(Fig. 6C)" at page 68, line 28; "(Fig. 6D1)" at page 68, line 30; "(Fig. 6D2)" at page 68, line 31; and "(6D3)" at page 69, line 3. In support of such an amendment to the specification, applicants respectfully direct the Examiner to the Brief Description of the Figures at page 4, lines 30-33 and page 5, lines 1-23 which describe as figures 6A-6C the effect of ABAD overexpression on the proton-decoupled 13c NMR spectra of freeze - clamped brain after perfusion with D-[2,4-^{13}C]β-hydroxybutyrate. Accordingly, applicants contend that the Brief Description of the

Figures at page 4, lines 30-33 and page 5, lines 1-23 describe the contents of figure 6, and as a summary of the content of figure 6 of the presently claimed invention, demonstrate that the inclusion of "(Fig. 6A)" at page 68, line 22; "(Fig. 6B)" at page 68, line 22; "(Fig. 6C)" at page 68, line 28; "(Fig. 6D1)" at page 68, line 30; "(Fig. 6D2)" at page 68, line 31; and "(6D3)" at page 69, line 3 was an obvious error and such amendment to the specification does not introduce any new matter.

The Examiner alleges that p.69 discusses NMR analysis and refers to Figures 7A, B, C and 8D. The Examiner alleges that the submitted figure 7 has parts A-D, and that there is no figure 8D.

In response, applicants have hereinabove amended the specification to correct an obvious error at pages 69-70. The specification no longer recites "(Fig. 7A)" at page 69, line 18; "(Fig. 7B)" at page 69, line 23; "(Fig. 7C)" at page 70, line 6; and "(Fig. 8D)" at page 70, line 10. In support of such an amendment to the specification, applicants respectfully direct the Examiner to the Brief Description of the Figures at page 5, lines 25-35 and page 6, lines 1-9 which describe as figures 7A-7D and 8 the induction of stroke in Tg PD-ABAD mice, and the identification of a double transgenic mouse overexpressing ABAD and mutant APP by PCR. Accordingly, applicants contend that the Brief Description of the Figures at page 5, lines 25-35 and page 6, lines 1-9 describe the contents of figures 7A-7D and 8, and as a summary of the contents of figures 7A-7D and 8 of the presently claimed invention, demonstrate that

the inclusion of "(Fig. 7A)" at page 69, line 18; "(Fig. 7B)" at page 69, line 23; "(Fig. 7C)" at page 70, line 6; and "(Fig. 8D)" at page 70, line 10 was an obvious error and such amendment to the specification does not introduce any new matter.

The Examiner alleged that p.70 discusses the induction of stroke in Tg PD-ABAD mice and refers to Figures 8A-D, yet allegedly there is only one Figure 8.

In response, applicants have hereinabove amended the specification to correct an obvious error at pages 70-71. The specification no longer recites "(Fig. 8A-B)" at page 70, line 26; "(Fig. 8C)" at page 70, line 32; and "(Fig. 8D)" at page 71, line 2. In support of such an amendment to the specification, applicants respectfully direct the Examiner to the Brief Description of the Figures at page 5, lines 25-35 and page 6, lines 8-9 which describe the identification of a double transgenic mouse overexpressing ABAD and mutant APP by PCR. Accordingly, applicants contend that the Brief Description of the Figures at page 6, lines 8-9 describe the contents of figures 8A-8D, and as a summary of the contents of figures 8A-8D of the presently claimed invention, demonstrate that the inclusion of "(Fig. 8A-B)" at page 70, line 26; "(Fig. 8C)" at page 70, line 32; and "(Fig. 8D)" at page 71, line 2 was an obvious error and such amendment to the specification does not introduce any new matter.

Applicants contend that the amended specification obviates the

Examiner's above objections and respectfully request that the Examiner reconsider and withdraw these grounds of objection

Rejection under 35 U.S.C. §112, first paragraph:

claims 1-10 and 14-16:

The Examiner rejected claims 1-10 and 14-16 under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. The Examiner alleged that the claimed invention is directed to a transgenic non-human animal whose cells contain a recombinant DNA sequence comprising a nerve tissue specific promoter operatively linked to a DNA sequence encoding amyloid-beta peptide alcohol dehydrogenase (ABAD), wherein said non-human animal exhibits elevated levels of basal amyloid precursor protein (APP), and methods of using the non-human animal in evaluation of potential therapeutic effects of compounds for the treatment of Alzheimer's disease. In further embodiments, the claimed invention is directed to a transgenic mouse whose cells contain a recombinant DNA sequence comprising a nerve tissue specific promoter and a DNA sequence which encodes amyloid-beta peptide alcohol dehydrogenase (ABAD), Wherein the mouse exhibits a phenotype consisting of overexpression of ABAD in the brain, increased ATP levels in the cerebral cortex and lower β-hydroxybutyrate levels in the cerebral cortex which has been subjected tp cerebral ischemia. The Examiner stated that the specification teaches methods of making a transgenic non-human

animal whose cells contain a recombinant DNA sequence comprising of a nervous tissue specific promoter operatively linked to a DNA sequence which encodes amyloid-beta peptide alcohol dehydrogenase (ABAD), and wherein the non-human animal exhibits the phenotype of elevated levels of basal ATP. The Examiner stated that this transgenic non-human animal can be used in evaluation of potential therapeutic effect(s) of an agent or compound in the treatment of Alzheimer's disease in a human (see p.8). The Examiner stated that the specification discusses general methods of generating transgenic non-human animals, embryonic target cells that would be used to introduce transgenes (such as zygotes for microinjection techniques and embryoinc stem(ES) cells), as well as methods to introduce transgenes into such cells(for example, retroviral infection) (see pp.24-29). The Examiner stated that the specification teaches the generation of transgenic mice with targeted overexpression of ABAD confers increased sensitivity to the toxic effects of Aβ. The Examiner alleged that to produce the transgenic mice, the platelet-derived growth factor (PDGF) B-chain promoter was operatively linked to the full-length human ABAD Cdna. A~3 kb fragment containing the promoter, cDNA and required sequences was then implanted into microinjected into mouse B6CBAF1/J oocytes. The Examiner alleged that these oocytes were then implanted into pseudopregnant females and mated with B6BAF1/J males, resulting in dependent founders. The Examiner alleged that breeding of these mice were then used to produce the Tg PD-ABAD mice which were then analyzed by northern analysis and western blotting (see p.34). The Examiner alleged that the Tg PD-ABAD mice

were then mated with Tg Happ mice, and the progeny were then characterized by analysis of brains for the studies of neuronal integrity (by laser scanning confocal microscopy) and degeneration. The Examiner alleged that the brains were divided sagitally and sectioned. The Examiner alleged that these sections were then immunostained (see pp.35-36). The Examiner alleged that the specification teaches that the Tg PD-ABAD/hAPP mice would be expected to show higher levels of ABAD and would be expected to show higher levels of ABAD and would potentially represent a model of the exaggerated effects of ABAD. The Examiner alleged that the Tg PD-ABAD/hAPP mice were observed for 3-4 months and the evidence of neuronal stress was then analyzed by studying the expression of 4-hydroxynonenal (HNE) (see p.47, and Figure 9). The Examiner alleged that the specification teaches that increased levels of HNE antigen were present in the Tg PD-ABAD/hAPP mice by 4 months of age when compared to single transgenic, and non-transgenic littermates. The Examiner alleged that the specification teaches that neuropathological studies, using markers associated with toxicity, were then undertaken to analyze whether the increased neuronal stress observed in the Tg PD-ABAD/hAPP mice would result in neuroprotection or neurotoxicity (see p.47, lines 15-33). The Examiner alleged that neurons from the Tg PD-ABAD mice were isolated and analyzed immunocytochemically using antibody to neurofilament-m and it was found that these cultured neurons displayed high levels of RAGE expression based upon Northern analysis and immunoblotting (see p.38 and p.48). The Examiner alleged that the specification teaches the generation of doubly

transgenic mice overexpressing ABAD and mutant APP (V717F, K670M, N671L). The Examiner alleged that basal synaptic transmission and synaptic plasticity was investigated in these mice and it was found that there was a 47% reduction if basal synaptic transmission in 10-month old mutant ABAD/APP mice when compared to wild type animals(see p.50), and that singly transgenic animals did not exhibit any change (see Example 2). The Examiner alleged that the specification further teaches that in the doubly transgenic mice overexpressing ABAD and mutant APP had evidence of neurotoxicity and the mice displayed increased neuronal stress (see Example 4). The Examiner stated that although the specification teaches methods to generate transgenic mice whose genome comprise a transgene encoding PDGF B-chain promoter operatively DNA sequence encoding ABAD, the specification fails to teach methods of generating any other transgenic non-human animals. The Examiner alleged that the specification fails to teach methods of generating any other transgenic non-human animals. The Examiner alleged that the specification fails to provide any relevant teachings or guidance with regard to the production of a transgenic non-human animal as claimed, and one of skill would not be able to rely on the state of the transgenic art for an attempt to produce transgenic non-human animals for the breadth claimed, which express a transgene encoding PDGF B-chain promoter operatively DNA sequence art for an attempt to produce transgenic non-human animals for the breadth claimed, which express a transgene encoding PDGF B-chain promoter operatively DNA sequence encoding ABAD. The Examiner alleged that this is because the art of transgenic animal product has for many

years stated that the unpredictability lies with the site or sites of integration of the transgene into the target genome. The Examiner stated that transgenic animals are regarded to have within their cells cellular mechanisms which prevent expression of the transgene, such as DNA methylation or deletion from the genome (Kappell et al (1992) Current Opinion in Biotechnology 3,549,col. 2, parag.2). The Examiner stated that Mullins et al.(1993) provide that not all animals express a transgene sufficiently to provide a model for a disease as the integration of a transgene sufficiently to provide a model for a disease as the integration of a transgene sufficiently to provide a model for a disease as the integration of a transgene sufficiently to provide a model for a disease as the integration of a transgene into difference species of animal has been reported to given divergent phenotypes (Mullins et al (1993) Hypertension 22, page 631, col. 1, parag. 1, parag.1, lines 14-17). The Examiner aleged that the elements of the particular construct used to make transgenic animals are held to be critical, and that they must be designed case by case without general rules to obtain good expression of a transgene; e.g., specific promoters, presence or absence of introns, etc. (Houdebine(1994) J.Biotech.34, page 281). The Examiner alleged that "The position effect" and unidentified control elements also are recognized to cause aberrant expression (Wall(1996) Theriogenology 45,61, parag. 2, line 9 to page 62, line 3). The Examiner stated that Mullins et al.(1996) disclose that "the use of nonmurine species for transgenesis will continue to reflect the suitability of a particular species for the specific questions being addressed, bearing in mind that a given

construct may react very differently from one species to another." (Mullins et al (1996) J. Clin. Invest. 98, page S39, Summary). The Examiner stated that well-regulated transgenic expression is not frequently achieved because of poor levels or the complete absence of expression or leaky expression in non-target tissues (Cameron (1997) Molec. Biol.7, page 256, col. 1-2, bridg. parag.). The Examiner stated that factors influencing low expression, or the lack their of, are not affected by copy number and such effects are seen in lines of transgenic mice made with the same construct(Cameron (1997), Molec. Biol. 7, page 256, lines 3-9). The Examiner stated that these factors, thus, are copy number independent and integration site dependent, emphasizing the role the integration site plays on expression of the transgene (Cameron (1997), Molec. Biol. 7, page 256, lines 10-13). The Examiner alleged that Sigmund (2000) discloses that the random nature of transgene insertion, resulting founder mice can contain the transgene at a different chromosomal site, and that the position of the transgene effects expression, and thus the observed phenotype (Sigmund (2000) Arteroscler. Throm. Vasc. Biol.20, page 1426, col.1, parag.1, lines 1-7). The Examiner stated that with regard to the importance of promoter selection, Niemann (1997) discloses that transgenic pigs made with different promoters regulating expression of a growth hormone gene give disparate phenotypes- one deleterious to the pig, the other compatible with pig health (Niemann (1997) Transg. Res. 7, page 73, col. 2, parag. 2, line 12 to page 73, col. 1, line 4). The Examiner stated that while the intent is not to say that transgenic animals of a particular

phenotype can never be made, the intent is to provide art taught reasoning as to why the instant claims are not enabled. The Examiner alleged that given such species differences in the expression of a transgene, particularly when taken with the lack of guidance in the specification for any transgene, particularly when taken with the lack of guidance in the specification for any transgenic non-human animal whose genome comprises a human ABAD transgene, other than the exemplified transgenic mouse, it would have required undue experimentation to predict the results achieved in any one host animal comprising and expressing human ABAD, the levels of the transgene product, the consequences of that product, and therefore, the resulting phenotype. The Examiner alleged that the specification does not contain a written description of any species of transgenic non-human animal of the type claimed, other than a mouse, as noted above. The Examiner alleged that no particular phenotype is disclosed for the claimed transgenic animals other than the anticipated expression of the transgene; and there is no demonstration that the claimed transgenic non-human animals would in fact express the transgene from the construct contemplated, nor that they would exhibit a phenotype useful for the claimed methods of use of the transgenic non-human animals. The Examiner aleged that without knowing the phenotype of a transgenic mouse, fish, cow, pig, bird, etc., one of skill in the art would not know how to use the animal. The Examiner alleged that specific phenotypic alterations cannot be predictably achieved by merely transferring a gene of interest into an animal, therefore, specific guidance must be provided to enable the instant

invention. The Examiner stated that the specification must teach those skilled in the art how to make and use the full scope of the claimed invention without undue experimentation. The Examiner alleged that it is known in the art that ABAD has been implicated in the pathogenesis of Alzheimer's disease by potentiating cell stress induced by amyloid beta peptide through observation of increased 4-hydroxynoneal-lysine, malondialdehyde-lysine epitopes and the induction DNA fragmentation (see p.53, lines 22-29). The Examiner stated that the specification discusses cases of severe metabolic stress, such as a murine stroke model in transgenic mice, in order to determine the consequences of overexpression of ABAD in cortical neurons(see p. 54, lines 21-34). The Examiner alleged that while the specification teaches that these mice exhibit increased ATP levels in the cerebral cortex, decreased lactate levels in the cerebral cortex and lower beta-hydroxbutyrate levels in the cerebral cortex which has been subjected to cerebral ischemia; however, the specification does not provide a nexus between these observed results and the claimed uses of this mouse, for example, methods for evaluating the potential therapeutic effect of a compound for the treatment of Alzheimer's disease in a human. The Examiner alleged that the specification has not provided any correlation between the observed results and Alzheimer's disease and furthermore, the transgenic non-human animal, as claimed, is indicated to show an increase in basal APP. The Examiner alleged that the specification does not provide a correlation between the exemplified transgenic mouse and an Alzheimer's disease model. The Examiner alleged that the

specification has not provided guidance as to how to make and use the claimed invention, as the specification only discloses the mice as Alzheimer's disease models, which would be used in evaluating therapeutic effects of an agent or compound for treatment of Alzheimer's disease (see p.8, lines 11-24). The Examiner alleged that the specification teaches that the loss of synaptophysin immunoreactivity in presynaptic terminals is associated with the Alzheimer's brain. The Examiner alleged that the specification teaches that immunostaining analysis of the hippocampus of the doubly-transgenic TgPD-ABAD mice with TghAPP mice with an antibody to synaptophysin demonstrated a reduction in the area of neuropil occupied by synaptophysin labeled presynatic terminals, and this would be consistent with evidence of neurotoxicity. The Examiner alleged that it was found that examination of older mice showed increased staining with an antibody selective for the activated form of caspase 3, which is additionally an evidence of neurotoxicity (see pp.47-48 and pp. 50-51). The Examiner alleged that while the specification teaches the generation of the doubly transgenic mice overexpressing three mutations of hAPP, it is not clear which of the Tg PD-ABAD/hAPP mice expressed the described reduction of synaptophysin, as such it is not clear what phenotype is observed with the described doubly-transgenic mice. The Examiner stated that in view of the alleged lack of guidance and direction provided by the specification for the generation and use of any other species of transgenic non-human animal, other than the exemplified Tg PD-ABAD mice, the alleged lack of guidance or teaching for methods of evaluating potential therapeutic effects of

compounds for treatment of Alzheimer's disease in humans using any transgenic non-human animal, the alleged unpredictable and undeveloped state of the art with respect to transgene behavior in transgenic animals of all species, the alleged lack of guidance or teaching provided by the specification for a correlation between the observed levels of the increased ATP levels in the cerebral cortex, decreased lactate levels in the cerebral cortex and lower betahydroxybutyrate levels in the cerebral cortex which has been subjected to cerebral ischemia, it would have required undue experimentation for one skilled in the art to generate the claimed transgenic non-human animals and methods of using the same.

In response, applicants respectfully traverse the Examiner's above rejection. Nevertheless, applicants without conceding the correctness of the Examiner's poistion but to expedite prosecution of the subject application have hereinabove canceled claims 3-4 and 7-8 without prejudice or disclaimer to applicants right to pursue the subject mater of this claim in a later-filed application and have amended claims 1-2, and 5. Claims 1-2 and 5 now recite "a transgenic mouse" and no longer recite the alleged limitation "a transgenic non-human animal." Further, amended claims 1-2, and 5 now recite "Alzheimer's disease-like pathology" rather than the alleged limitation "Alzheimer's disease." Accordingly, applicants contend that amended claims 1-2 and 5 obviate the Examiner's above rejection and respectfully request that the Examiner reconsider and withdraw these grounds of rejection.

Claims 11-14:

The Examiner rejected claims 11-14 under 35 U.S.C. §112, first paragraph, alleging that the specification, while being enabling for an in vitro method for increasing a cell's resistance to metabolic stress in the absence of glucose and the presence of β-hydroxybutyrate, the method comprising transfecting into the cell a DNA construct encoding a promoter operatively linked to a DNA sequence encoding ABAD, wherein expression of ABAD maintains cellular metabolism, thus increasing the resistance of the cell to metabolic stress, does not reasonably provide enablement for all methods for increasing resistance to metabolic stress. The Examiner stated that the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention commensurate in scope with these claims. The Examiner stated that the claimed invention is directed to a method for increasing a cell's resistance to metabolic stress comprising introducing into the cell a recombinant DNA molecule which comprises a promoter operatively linked to a DNA sequence encoding ABAD wherein the cell expresses ABAD, thereby increasing the basal level of ATP in the cell and increasing the resistance of the cell to metabolic stress. The Examiner alleged that the specification teaches that using recombinant ABAD that was produced in E.coli was purified and the metabolism of β-hydroxybutyrate and β-hydoxybuyrl-CoA was measured (see p. 55 and p.63). The Examiner alleged that it was found that in a purified system, ABAD is more effective with β-hydroxbutyryl-CoA as a substrate (see p.64, line 5); and that ABAD would have the

potential for enhancing the metabolism of β-hydroxybutyrate. The Examiner alleged that the specification teaches that COS cells were transfected with pcDNA3/human wild-type ABAD (wtABAD), or mutant ABAD (mutABAD), OR VECTOR ALONE (SEE P. 55-56 AND PP.64-65). The Examiner alleged that to assay cellular function, these cells were placed in a medium devoid of glucose, which was supplemented with dialyzed serum and β-hydroxybutyrate, and it was found that the Cos/vector and Cos/mutABAD cells had compromised cellular functions. The Examiner alleged that in contrast, COS/wtABAD cells maintained better MTT reduction and cellular energy charge (See p.66, lines 5-10), furthermore, it was shown that the changes in cellular properties were paralleled by the maintenance of the morphological phenotype of the COS/wt ABAD cells, which did not become rounded up and toxic in appearance, as seen in the Cos/vector and Cos/mutABAD cells. The Examiner alleged that the specification further teaches that NMR studies were performed on COS/wtABAD cells incubated with [13C]-D-β-hydroxybutyrate to determine the effect of ABAD on the metabolism of these cells in medium devoid of glucose (see pp.66-67). The Examiner alleged that as the labeled [13C]-D-β-hydroxybutyrate entered the TCA cycle, the [13C-2] is metabolized to α-ketoglutarate entered the TCA cycle, the [13C-2] is metabolized to a-ketoglutarate, which is in rapid equilibrium with glutamate. The Examiner alleged that it was found through the NMR studies that there was a 2-fold greater labeling of glutamate in COS/wtABAD cells when compared to COS/vector cells. 1H-NMR analysis showed that the supernatants of COS/wtABAD transfectant cells, there was ~58% fractional enrichment at C-4,

when compared to ~41% in COS/vector cells. The Examiner alleged that this data is indicative of increased metabolism of the exogenous [13C]-D-β-hydroxybutyrate in the COS/wtABAD transfected cells. The Examiner alleged that the specification teaches NMR measurements that are based upon the conversion of α-ketoglutarate to glutarate in COS cells in vitro. The Examiner alleged that the specification discusses in vivo applications of the claimed methods, such as the transfection of ABAD genes into pancreatic islet cells in order to see enhanced viability after transplantation (see p. 17-18, bridging paragraph). The Examiner alleged that the specification discusses in vivo applications of the claimed methods, such as the transfection of ABAD genes into pancreatic islet cells in order to see enhanced viability after transplantation (see p. 17-18, bridging paragraph). The Examiner alleged that the specification is enabling for increasing a cell's resistance to metabolic stress in vitro, but that the specification does not provide guidance or teaching to enable such a method in vivo. The Examiner alleged that this is because in general, in vitro gene expression is not representative of gene expression in a host subject whose cells(or target cells) have been somatically transfected in vivo. The Examiner alleged that this is because numerous factors complicate in vivo gene transfer and expression which result in therapeutic effects. The Examiner cited Eck & Wilson ('Gene-Based Therapy' in the Pharmacological Basis of Therapeutics , 1996), who report that numerous factors complicate in vivo gene therapy with respect to predictably achieving levels and duration of gene expression which have not been shown to be

overcome by routine experimentation. The Examiner alleged these include, the fate of DNA vector itself (Volume distribution, rate of clearance into the tissue, etc.), the in vivo consequence of altered gene expression and protein function, the fraction of vector taken up by the target cell population, the trafficking of the genetic material within cellular organelles, the rate of degradation of DNA, the level of mRNA produced, the stability of the mRNA produced, the amount and stability of the protein produced, and the protein's compartmentalization within the cell, or its secretory fate, once produced. The Examiner alleged that these factors differ dramatically based on the vector used, the route of administration of the vector, the protein being produced, which cells are the target cells, and the disease and/or host being treated. The Examiner alleged that Eck and Wilson support the importance of tailoring a gene therapy vector and method to specific disease and/or disorders. The Examiner alleged that Eck & Wilson et al. review the state of the art for gene therapy for inherited disorders and discloses that "[t]he level of protein function necessary to achieve complementation of the defect varies widely among genetic disease." The Examiner alleged that the specification fails to address how to overcome any of the above described unpredictable parameters in the gene therapy art such that one skill in the art would be able to achieve increased cellular resistance to metabolic stress, nor does the specification teach which cells would be the target cells for the claimed method, and how such target cells would be contacted (for example, what route of administration?). The Examiner alleged that with respect

to the specification's lack of teaching or sufficient guidance for ABAD expression in vivo to increase a cell's resistance to metabolic stress, it is not predictable if ABAD gene expression would start or continue in target cells, or any cells, at levels and for a duration which would be considered therapeutic because somatic gene delivery often results in only limited expression in inadequate numbers of cells. The Examiner alleged that in view of the quantity of experimentation necessary to determine the parameters listed above for ABAD gene therapy, the lack of direction or guidance provided by the specification to carry out ABAD gene therapy, the absence of working examples for the demonstration or correlation to achieving ABAD gene therapy in vivo, as well as the unpredictable and undeveloped state of the art with respect to the gene therapy art, it would have required undue experimentation for one skilled in the art to carry out the claimed methods.

In response, applicants respectfully traverse the Examiner's above rejection. Nevertheless, applicants without conceding the correctness of the Examiner's poistion but to expedite prosecution of the subject application have hereinabove canceled claims 11-13 without prejudice or disclaimer to applicants' rights to pursue the subject matter of this claim in a later-filed application. Accordingly, applicants contend that canceled claims 11-13 obviate the Examiner's above rejection and respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Rejection under 35 U.S.C. §112, second paragraph:

The Examiner alleged that claim 1, as written, is vague and confusing. The Examiner stated that the claim recites the phrase, "A transgenic non-human animal whose cells contain" in line 1 of the claim. The Examiner alleged that it is not clear how the cells "contain" a recombinant DNA sequence. The Examiner suggested that the claim be written to state, "A transgenic non-human animal genome comprises." The Examiner States claims 2-4 depend from claim 1. The Examiner alleged that claim 5, as written, is vague and confusing. The Examiner stated that the claim recites the phrase, "A transgenic non-human animal whose cells contain" in part (a) of the claim. The Examiner alleged that it is not clear how the cells "contain" a recombinant DNA sequence. The Examiner suggested that the claim be written to state, "A transgenic non-human animal whose genome comprises." The Examiner States claims 6-10 depend from claim 5.

In response, applicants respectfully traverse the Examiner's above rejection. Nevertheless, applicants without conceding the correctness of the Examiner's poistion but to expedite prosecution of the subject application have hereinabove amended claim 1. Claim 1 now recite "whose genome comprise" and no longer recites the alleged limitation "whose cells contain." Accordingly, applicants contend that amended claim 1 obviates the Examiner's above rejection and respectfully request that the Examiner reconsider and withdraw this ground of rejection.

The Examiner alleged that the term "essentially" in claim 9 is a relative term which renders the claim indefinite. The Examiner alleged that the term "essentially" is not defined by the claim, the specification does not provide a standard for ascertaining the requisite degree, and one of ordinary skill in the art would not be reasonably apprized of the scope of the invention. The Examiner stated that clarification and/or amendment is requested.

In response, applicants respectfully traverse the Examiner's above rejection. Nevertheless, applicants without conceding the correctness of the Examiner's poistion but to expedite prosecution of the subject application have hereinabove canceled claim 9 without prejudice or disclaimer to applicants' right to pursue the subject matter of this claim in a later-filed application. Accordingly, applicants contend that cancelled claim 9 obviates the Examiner's above rejection and respectfully request that the Examiner reconsider and withdraw this ground of rejection.

The Examiner alleged that claim 11, as written, is vague. The Examiner alleged that the claim recites the term, "introducing" in line 2 of the claim, but that this term is not defined by the claim. The Examiner alleged that it is not clear how the recombinant DNA molecule is introduced into the cell (e.g., injection, transfection, etc). The Examiner stated that clarification and/or amendment is requested. The Examiner stated that claims 12-13 depend from claim 11. The Examiner alleged that claim 11, as written, is incomplete. The Examiner stated that the

claim recites the phrase, "wherein the cell expresses ABAD, thereby increasing the basal level of ATP in the cell and increasing the resistance of the cell to metabolic stress." The Examiner alleged that it is not clear how the mere expression of ABAD would increase the basal level of ATP. The Examiner stated that clarification and/or amendment is requested. The Examiner stated that claims 12-13 depend from claim 11. The Examiner alleged that claim 12, as written is confusing. The claim recites the term, "excitotoxic stress" in line 2 of the claim. It is not clear what this term encompasses. Clarification and/or amendment is requested.

In response, applicants respectfully traverse the Examiner's above rejection. Nevertheless, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have hereinabove canceled claim 11 without prejudice or disclaimer to applicants' right to pursue the subject matter of this claim in a later-filed application. Accordingly, applicants contend that canceled claim 11 obviates the Examiner's above rejection and respectfully request that the Examiner reconsider and withdraw this ground of rejection.

The Examiner stated that claim 14, as written, is vague and confusing. The claim recites the phrase, "A transgenic mouse whose cells contain" in line 1 of the claim. The Examiner alleged that it is not clear how the cells "contain" a recombinant DNA sequence, nor it is clear which cells contain the DNA sequence. The Examiner suggested that the claim be written to state, "a transgenic mouse

whose genome comprises..." The Examiner stated that claims 15-16 depend from claim 14.

In response, applicants respectfully traverse the Examiner's above rejection. Nevertheless, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have hereinabove amended claim 14. Claim 14 now recite "whose genome comprise" and no longer recite the alleged limitation "whose cells contain." Accordingly, applicants contend that amended claim 14 obviates the Examiner's above rejection and respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Rejection under 35 U.S.C. §103:

The Examiner rejected claims 1, 3, 4, 14 and 16 under 35 U.S.C. §103 (a) as being unpatentable over Moechars et al. (JBC, Vol. 274, March 1999, pp. 6483-6492) when taken with He et al. (JCB, Vol. 273, April 1998, pp. 10741-10746). The Examiner alleged that Moechars et al. teach the generation of transgenic mice that overexpress different mutations of amyloid precursor protein (APP) in their brains(See Abstract). The Examiner alleged that Moechars et al. teach that these mice demonstrated increased Aβ levels which correlated with the formation of amyloid plaques in the brains of old APP/London transgenic mice, and after analysis of the plaques, it was found that they displayed many aspects typically observed in the brain of Alzheimer's disease (see p. 6483, col. 2, 2nd paragraph). The Examiner alleged the transgenic mice were

generated using cDNA coding for various APP forms (both wild-type and mutant), where the cloned and linearized minigenes were microinjected into prenuclear embryos, see p. 6483, 2nd column. The Examiner alleged that Moechars et al. teach that the transgene was driven by the mouse thy-1 promoter (a nervous-tissue specific promoter) (see p.6484, 2nd column, Results). The Examiner stated that Moechars et al. differ from the claimed invention in that they do not use a targeting construct containing human ABAD. The Examiner alleged that prior to the time of filing, He et al. teach the cloning and characterization of ABAD (also known ERAB). The Examiner alleged that He et al. teach the sequences of ABAD (see Figure 2) and discuss its role in mediating neurotoxicity in Alzheimer's disease (see Abstract). The Examiner stated that absent any phenotypic requirements of the claimed transgenic non-human animal, the combination of the cited prior art is sufficient to make obvious the claimed invention. The Examiner alleged that in view of the teachings of He et al., it would have been obvious for one ordinary skill in the art, at the time the claimed invention was made, to generate transgenic mice with a transgene under the control of the thy-1 promoter as described by Moechars et al. with a transgene encoding ABAD, with a reasonable expectation of success. The Examiner alleged that one of ordinary skill in the art would have been sufficiently motivated to make such a modification, as asserted by He et al., that because ABAD mediates neurotoxicity in Alzheimer's disease, there would be a need to examine it's function in the pathogenesis of the disease. The Examiner alleged that the claimed invention as a whole was clearly

prima facie obvious at the time the claimed invention was made especially in the absence of sufficient, clear and convincing evidence to the contrary.

In response, applicants respectfully traverse the Examiner's above rejection. Applicants contend that it would not have been obvious to one of skill in the art to combine the mouse thy-1 promoter of Moechars et al. with the ABAD sequences of He et al. to create the present invention because the prior art references neither teach nor suggest the transgenic mouse of the present invention exhibiting at least one phenotype from the group consisting of: overexpression of ABAD; elevated levels of basal ATP; protection from metabolic or ischemic stress. Therefore, the prior art references do not provide a suggestion or motivation to modify the reference teachings to produce the claimed invention. Accordingly, the claimed invention was not obvious in view of Moechars et al. taken with He et al. Should the Examiner disagree, it is respectfully requested that the Examiner specify where in the cited prior art documents there is a basis for such disagreement.

Summary

For the reasons set forth hereinabove, applicants respectfully request that the Examiner reconsider and withdraw the various grounds of objection and rejection and earnestly solicit allowance of the now pending claims, i.e. claims 1-2, 5-6, 10 and 14-16.

If a telephone interview would be of assistance in advancing

prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone either of them at the number provided below.

No fee, other than the enclosed $460.00 fee for a three-month extension of time, is deemed necessary in connection with the filing of this Amendment. However, if any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,

I hereby certify that this correspondence is being deposited this date with the U.S. postal Service with sufficient postage as first class mail in an envelope addressed to:

Assistant Commissioner of Patents
Washington, DC 20231

6/20/02
Alan J. Morrison Date
Reg. No. 37,399

John P. White
Registration No. 28,678
Alan J. Morrison
Registration No. 37,399
Attorneys for Applicants
Cooper & Dunham LLP
1185 Avenue of the Americas
New York, New York 10036
(212) 278-0400

Exhibit A

at page 8, after line 2:

--The present invention provides for a transgenic non-human animal whose cells contain a recombinant DNA sequence comprising a nerve tissue specific promoter operatively linked to a DNA sequence which encodes amyloid-beta peptide alcohol dehydrogenase (ABAD), wherein said non-human animal exhibits at least one phenotype from the group consisting of: overexpression of ABAD, elevated levels of basal ATP; protection from metabolic or ischemic stress.--

at page 15, after line 25:

-- The transgenic non-human mammals of the present invention will provide insights with respect to how and where protein interactions occur in Alzheimer's Disease and thus provide more useful models for testing the efficacy of certain drugs in preventing or reducing the onset or progression of this disease. The transgenic non-human mammals of the present invention include recombinant genetic material comprised of a nucleic acid sequence encoding [RAGE] ABAD fused to specific promoters capable of expressing the protein in specific tissues such as nerve tissues generally and/or specific types of nerve tissue, e.g., the brain.--

at page 17, after line 25:

-- In addition, the ABAD gene has been introduced into pancreatic islet [cels] cells in order to see if it would enhance their viability after transplantation. In this

case, survival means that few islet cells would be needed for a particular transplant recipient for the tranplant to be successful and accordingly, the surviving islet cells being able to produce insulin. This is of great value to patients who are islet transplant recipients since the problem in islet cell transplantation is that there are too few islet cells available and if the number of islet cells per transplant were reduced, then the number of transplants possible would increase. Therefore, it is clear that one property exhibited by the ABAD overexpressing transgenic and by the islet cells which have ABAD expressed via introduction of a recombinant expression construct (DNA including a sequence encoding ABAD operatively linked to a promoter sequence and integrated into the genome of the islet cells) is that of cytoprotection. This cytoprotection is due, at least in part, to the consequence that overexpression of ABAD (as shown in the phenotype of the ABAD transgenic) results in an increase in cellular ATP.--

at page 34, after line 28:

-- Cross-breeding of Tg [PD-RAGE] PD-ABAD mice with Tg hAPP mice. Tg mice overexpressing an alternatively spliced hAPP minigene that encodes hAPP695, hAPP751, and hAPP770 bearing mutations linked to familial AD (V717F, K670M/N671L) have been produced by Dr. Lennart Mucke[14], and provided to us for use in cross-breeding studies with Tg [PD-RAGE] PD-ABAD mice. In these mice, expression of the transgene is also driven by the PDGF B-chain

promoter. Cross-breeding was performed and double-transgenic mice expressing both hAPP and PD-ABAD transgenes were identified with specific primers. The primers for the hAPP transgene were: 5'-GACAAGTATCTCGAGACACCTGGGGATGAG-3' **(SEQ ID NO:6)** and 3'-AAAGAACTTGTAGGTTGGATTTTCGTACC-5' **(SEQ ID NO:7)**. PCR conditions for the amplifying the hAPP transgene were the same as those described above, and the size of the amplicon was 1169 bp.--

page 41, after line 14:

-- Induction of stroke in Tg [PD-RAGE] PD-ABAD mice. Functional consequences of overexpression of [RAGE] ABAD were first assessed in response to ischemic stress, the transient middle cerebral artery occlusion model. *Murine stroke model* Mice (C57BL6/J, male) were subjected to stroke according to previously published procedures[32]. Following anesthesia, the carotid artery was accessed using the operative approach previously described in detail[33], including division/coagulation of the occipital and pterygopalatine arteries to obtain improved visualization and vascular access. A nylon suture was then introduced into the common carotid artery, and threaded up the internal carotid artery to occlude the origin of the right middle cerebral artery (MCA). Nylon (polyamide) suture material was obtained from United States Surgical Corporation (Norwalk, CT), and consisted of 5.0 nylon/13 mm length for 27-36 g mice, and 6.0 nylon/12 mm length for 22-26 g mice. After 45 minutes of occlusion, the suture was withdrawn to achieve a

reperfused model of stroke. Although no vessels were tied off after the suture was removed, the external carotid arterial stump was cauterized to prevent frank hemorrhage.--

page 48, after line 3:

-- Characterization of neurons isolated from Tg PD-ABAD mice. Neuronal cultures were made from the cerebral cortex from E16 mouse embryos. These cultures were >90% neurons based on staining with anti-neurofilament antibody (not shown). Cultured neurons displayed high levels of [RAGE] ABAD expression based on Northern analysis and immunoblotting [(Fig. **13**)].--

page 61, after line 20:

-- Murine stroke model. Mice (C57BL6/J, male) were subjected to stroke according to previously published procedures (45) and in accordance with guidelines of the American Academy of Accreditation of Laboratory Animal Care (AAALAC). Following anesthesia, the carotid artery was accessed using the operative approach previously described in detail (46), including division/coagulation of the occipital and pterygopalatine arteries to obtain improved visualization and vascular access. A nylon suture was then introduced into the common carotid artery, and threaded up the internal carotid artery to occlude the origin of the right middle cerebral artery (MCA). Nylon (polyamide) suture material was obtained from United States Surgical Corporation (Norwalk, CT), and consisted of 5.0 nylon/13 mm length for 27-36 g mice,

and 6.0 nylon/12 mm length for 22-26 g mice. After 45 minutes of occlusion, the suture was withdrawn to achieve a reperfused model of stroke. Although no vessels were tied off after the suture was removed, the external carotid arterial stump was cauterized to prevent frank hemorrhage. Measurements of *relative cerebral blood flow* were obtained as previously reported (45-48) using a straight laser doppler flow probe placed 2 mm posterior to the bregma, and 6 mm to each side of midline using a stereotactic micromanipulator, keeping the angle of the probe perpendicular to the cortical surface. These cerebral blood flow measurements, expressed as the ratio of ipsilateral to contralateral blood flow, were obtained at baseline, and immediately prior to MCA occlusion, 45 minutes after MCA occlusion, and at several time points after withdrawal of the occluding suture. *Measurement of Cerebral Infarction Volumes*: After 24 hours, animals were euthanized and their brains rapidly harvested. Infarct volumes were determined by staining serial cerebral sections with triphenyl tetrazolium chloride and performing computer-based planimetry of the negatively (infarcted) staining areas to calculate infarct volume (using NIH image software). *Neurological Exam*: [ ]Prior to giving anesthesia, mice were examined for neurological deficit 23 h after reperfusion using a four-tiered grading system: a score of 1 was given if the animal demonstrated normal spontaneous movements; a score of 2 was given if the animal was noted to be turning towards the ipsilateral side; a score of 3 was given if the animal was observed to spin longitudinally

(clockwise when viewed from the tail); and, a score of 4 was given if the animal was unresponsive to noxious stimuli. This scoring system has been previously described in mice (45-47), and is based upon similar scoring systems used in rats (49). Immunostaining of cerebral cortex following induction of stroke in wild-type mice was performed as described above using a rabbit polyclonal antibody made using purified recombinant murine ABAD as the immunogen. Quantitation of microscopic images was accomplished with the Universal Imaging System.--

page 65, after line 22:

-- ABAD and the response of COS cells to nutritional stress. In view of ABAD metabolism of D-ß-hydroxybutyrate, we tested whether ABAD-transfected COS cells displayed enhanced ability to sustain nutritional stress in an environment where ketone bodies provided the principal energetic source. When COS/vector cells were placed in medium devoid of glucose and supplemented only with dialyzed serum and ß-hydroxybutyrate, cellular functions became compromised. In the presence of D-ß-hydroxybutyrate (10 mM), reduction of MTT was suppressed by days 4-5 [(Fig. **3A**)] and cellular energy charge decreased in parallel [(Fig. **3C**)]. Phase contrast microscopy showed COS/vector cells, initially with a spread morphology on the growth substrate, to become rounded up and toxic in appearance after four days under these conditions [(Fig. **3DI-II**)]. Similar results were obtained when COS/vector cells were replaced with

wild-type COS cells, and the same studies described above were performed (not shown). In contrast, COS/wtABAD cells better maintained MTT reduction [(Fig. 3A)] and cellular energy charge [(Fig. 3C)] in the presence of ß-hydroxybutyrate. These changes in cellular properties were parallelled by maintenance of the morphologic phenotype of COS/wtABAD cells, compared with COS/vector transfectants [(Fig. 3DIII-IV)] in the presence of ß-hydroxybutyrate. The effect of ß-hydroxybutyrate to maintain cellular functions in COS/wtABAD cells was dose-dependent, as shown using the MTT reduction assay, and reached a plateau by 10 mM [(Fig. **3B**)]. Furthermore, experiments with lower concentrations of ß-hydroxybuyrate (_2.5 mM) displayed less effective maintenance of cellular properties with COS/wtABAD cells (not shown). The requirement for enzymatically intact ABAD (wtABAD) for enhanced survival of COS/wtABAD cells in the presence of ß-hydroxybutyrate was shown by experiments performed with COS/mutABAD cells. Though the crippled enzyme (mutABAD) was expressed at similar levels and with a similar subcellular distribution as the wild-type enzyme (the latter in COS/wtABAD cells), COS/mutABAD cells responded to glucose replacement with ß-hydroxybutyrate as did COS/vector cells; there was a steady decline in MTT reduction and cellular energy charge [(Fig. 3A,C)].--

page 66, after line 29:

-- NMR studies were performed on cells incubated with [^{13}C]-D-ß-hydroxybutyrate (labelled in the C-2 and C-4 positions) to determine the effect of ABAD on the

metabolism of the COS/wtABAD transfectants in medium devoid of glucose. [^{13}C]-labelled ß-hydroxybutyrate enters the TCA cycle as [^{13}C-2]-labelled acetyl-CoA and is metabolized to α-ketoglutarate. Thus, C-4 gets labelled in the first turn of the TCA cycle, and, subsequent, labelling occurs in the C-3 and C-2 positions. As α-ketoglutarate is in rapid equilibrium with glutamate, the labelling of [^{13}C]-glutamate in the C-4, 3 and 2 positions was observed [(Fig. **4A**)]. Since the flux of [^{13}C] labelled acetyl-CoA via the TCA cycle is orientation conserved, the labelling of [^{13}C]-glutamate is greater in the C-4, compared with the C-3 and C-2, positions. Thus, labelling was evaluated in the C-4 position of glutamate in COS/wtABAD versus COS/vector in cell lysates and supernatants. In culture supernatants, NMR data demonstrated greater labelling of the C-4 resonances in glutamate in COS/wtABAD cells, _2-fold, compared with COS/vector cells [(Fig. **4B**; $p<0.03$ at days 4, 6 and 8)]. Glutamine was not detected in supernatants from COS/wtABAD or COS/vector cells. The ^{1}H NMR analysis of these supernatants revealed that the fractional enrichment at glutamate C-4 was 58±3% in COS/wtABAD transfectants compared with 41±2% in COS/vector cells. These data are indicative of increased metabolism of exogenous [^{13}C]-labelled ß-hydroxybutyrate in COS cells overexpressing ABAD. In contrast, there were no observed differences between fractional enrichment at glutamate C-4 in cell lysates between COS/wtABAD and COS/vector cells, probably because glutamate is rapidly extruded into the medium (glutamine

and glutamate levels in cell lysates were the same, comparing COS/wtABAD and COS/vector cells). The ^{13}C and ^{1}H NMR data demonstrate increased exogenous ß-hydroxybutyrate utilization in the COS cells overexpressing wtABAD.--

page 67, after line 32:

-- Upregulation of ABAD in response to cerebral ischemia. A severe form of metabolic stress is imposed by cerebral ischemia. Wild-type C57BL6 mice subjected to transient middle cerebral artery occlusion displayed increased levels of ABAD in neurons near the infarcted area [(Fig. **5A**)], especially those in the penumbra (Fig. 5A, *inset*), compared with the nonischemic hemisphere [(Fig. **5B**)], using polyclonal antibody to recombinant mouse ABAD. Image analysis of multiple fields from sections similar to those shown in panels A-B demonstrated an _5-fold increase of ABAD antigen in cortical neurons consequent to stroke [(Fig. **5C**)].--

page 68, after line 10:

-- Characterization of Tg PD-ABAD mice. These data with wild-type mice subjected to cerebral ischemia suggested that upregulation of ABAD might be a component of the response to ischemic stress, and led us to make transgenic mice in which ABAD was overexpressed in cortical neurons. Three independent founders of Tg mice in which human ABAD is expressed under control of the human PDGF B-chain promoter have been identified and used to establish transgenic lines (at present backcrossed

eight times into the C57BL6 background). Representative mice from each of these transgenic lines showed high levels of transgene activity at both the mRNA [(Fig. **6A**)] and protein levels [(Fig. **6B**)] in cerebral cortex. Immunoblotting performed on brain subregions from one line of Tg PD-ABAD mice, using an anti-human ABAD peptide antibody which selectively recognizes the human form of the protein, showed increased antigen especially in cerebral cortex and hippocampus, with a smaller increase in cerebellum [(Fig. **6C**)]. Immunohistochemical staining of ABAD in cerebral cortex confirmed high levels of antigen expression in cortical neurons [(Fig. **6D1**)] compared with nontransgenic littermates [(Fig. 6D2)]. Semiquantitative analysis of immunohistochemical results using antibody reactive with murine and human ABAD antigen (i.e., total ABAD antigen) indicated that there was an _3.5-4-fold increase in total ABAD antigen in cerebral cortex comparing Tg mice with nonTg littermate control mice [(Fig. 6D3)]. Induction of stroke in transgenic mice further elevated ABAD antigen another two-fold compared with nonTg controls (24 hrs after the ischemic episode; not shown). Growth (height/weight) and reproductive fitness (number and size of litters) was similar between Tg PD-ABAD mice and nontransgenic (nonTg) controls, and there were no overt neurologic symptoms or other phenotype evident in these mice noted to date.--

page 69, after line 12:

-- NMR analysis of ^{13}C-ß-hydroxybutyrate metabolism in Tg PD-ABAD mice. Tg PD-ABAD and control mice were infused

with D-[2,4-^{13}C]-3-hydroxybutyrate. ^{13}C NMR spectra of cerebral cortical extracts from Tg PD-ABAD and nonTg littermate control (the latter spectra are not shown) mice illustrate labelling of glutamate and glutamine in the C-4, C-3 and C-2 positions, as well as GABA in the C-2 position [(Fig. **7A**)], consistent with entry of [^{13}C]-ß-hydroxybutyrate via 2-[^{13}C]-acetyl-CoA into the TCA cycle. The intensity of glutamate and glutamine C-4 resonance was 50% and 20% greater, respectively, in Tg PD-ABAD mice compared with nonTg littermates [(Fig. **7B**)]. The glutamate to glutamine ratio, based on C-4 resonance area, was 3.6 ± 0.3 in nonTg versus 2.1 ±0.4 in Tg PD-ABAD mouse brains ($P<0.03$). These data suggest that glutamine synthesis is more efficient in Tg PD-ABAD mouse brain compared with controls. The area of the ^{13}C-labelled C-2 resonance of GABA was also greater in Tg PD-ABAD mice (4.8±0.3) than in nonTg controls (2.2±0.5; $p<0.04$). Such increased labelling of GABA is consistent with enhanced conversion of labelled glutamate to GABA in brains of Tg mice. ^{1}H NMR analysis of these extracts revealed that the fractional enrichment in glutamate C-4 was significantly greater in Tg PD-ABAD (58 ± 5%) than in nonTg littermates (38 ± 7%; $P<0.03$). As might be expected from the increased utilization of exogenous ß-hydroxybutyrate, measurement of basal ATP levels in cerebral cortex of Tg PD-ABAD mice fasted overnight showed a statistically significant increase compared with nonTg littermates [(Fig. **7C**)]. Similarly, levels of ß-hydroxybutryate in the brains of Tg PD-ABAD mice were lower, compared with nonTg controls in view of its

increased utilization in the presence of higher levels of neuronal ABAD [(see Fig. **8D**, animals not subjected to stroke)].--

page 70, after line 12:

-- Induction of stroke in Tg PD-ABAD mice. In order to assess the possible contribution of ABAD to ischemic stress, Tg PD-ABAD and age-matched nonTg littermate control mice were subjected to a 45 min period of transient middle cerebral artery occlusion followed by a 24 hr period for evolution of the cerebral infarct. These studies utilized a protocol which we have found to provide reproducible stroke volumes and functional data (neurologic deficit scores, cerebral blood flow)(45-48). Levels of ABAD increased an additional _2-fold in Tg PD-ABAD mice after stroke, versus with uninstrumented Tg PD-ABAD mice. Compared with nonTg littermate controls, Tg PD-ABAD mice displayed strokes of smaller volume and lower neurologic deficit scores (consistent with better maintenance of neurologic function)[(Fig. **8A-B**)]. In contrast, there was no change in cerebral blood flow comparing the Tg and nonTg animals (not shown), consistent with a direct effect of ABAD on neurons, as ABAD was overexpressed in neurons by the PDGF B chain promoter. Analysis of cerebral cortex from Tg animals showed increased ATP and decreased lactate levels compared with nonTg controls [(Fig. **8C**)]. In addition, ß-hydroxybutyrate levels were lower in animals subjected to cerebral ischemia, and this finding was much more

pronounced in Tg PD-ABAD mice compared with nonTg littermate controls [(Fig. **8D**)]. These data are suggested better maintenance of energy reserve and substrate metabolism in Tg PD-ABAD mice subject to ischemia.--

page 96, after line 2:

-- **Transgenic Mice Over-expressing Amyloid Beta Alcohol Dehydrogenase (ABAD) in Brain as Model of Alzheimer's Disease and Uses Thereof**

Abstract of the Disclosure

The present invention provides for a transgenic non-human animal whose cells contain a recombinant DNA sequence comprising a nerve tissue specific promoter operatively linked to a DNA sequence which encodes amyloid-beta peptide alcohol dehydrogenase (ABAD), [wherein said non-human animal] and exhibits at least one phenotype from the group consisting of: overexpression of ABAD, elevated levels of basal ATP; protection from metabolic or ischemic stress.--

Exhibit B

--1. (Amended) A transgenic [non-human animal] mouse whose [cells contain] genome comprise a recombinant DNA sequence comprising a nerve tissue specific promoter operatively linked to a DNA sequence encoding amyloid-beta peptide alcohol dehydrogenase (ABAD), wherein said [non-human animal] transgenic mouse exhibits elevated levels of basal amyloid precursor protein (APP).--

--2. (Amended) The transgenic [non-human animal] mouse of claim 1, wherein the promoter is platelet derived growth factor (PDGF)-B-chain promoter.--

--5. (Amended) A method for evaluating in a [non-human] transgenic [animal] mouse the potential therapeutic effect of a compound for treating Alzheimer's disease-like pathology in a human, which comprises:

(a) administering the compound to a transgenic [non-human animal] mouse whose [cells] genome comprise a recombinant DNA sequence comprising a nerve tissue specific promoter operatively linked to a DNA sequence which encodes amyloid-beta peptide alcohol dehydrogenase (ABAD), and

(b) determining the therapeutic effect of the compound on the transgenic [non-human animal]

mouse by monitoring basal synaptic transmission or synaptic plasticity or basal levels of ATP, wherein an increase in basal synaptic transmission or synaptic plasticity or basal levels of ATP indicates that the compound would have a potential therapeutic effect on Alzheimer's disease-like pathology in a human.--

--14. (Amended) A transgenic mouse whose [cells contain] genome comprise a recombinant DNA sequence comprising:

(a) a nerve tissue specific promoter; and

(b) a DNA sequence which encodes amyloid-beta peptide alcohol dehydrogenase (ABAD),

wherein the promoter and the DNA sequence which encodes amyloid-beta peptide alcohol dehyrdogenase are operatively linked to each other and integrated in the genome of the mouse, and

wherein said mouse exhibits at least one phenotype from the group consisting of: overexpression of ABAD in brain, increased ATP level in cerebral cortex; decreased lactate level in cerebral cortex and lower beta-hydroxybutyrate levels in cerebral cortex which has been subjected to cerebral ischemia.--

ELSEVIER

Developmental Brain Research 132 (2001) 121–129

DEVELOPMENTAL BRAIN RESEARCH

www.elsevier.com/locate/bres

Research report

Multiple pathways of neuroprotection against oxidative stress and excitotoxic injury in immature primary hippocampal neurons

Lynn M. Almli[a], Shannon E.G. Hamrick[a], Anita A. Koshy[a], Martin G. Täuber[b], Donna M. Ferriero[a,*]

[a]*Departments of Neurology and Pediatrics, University of California San Francisco, Box 0114, San Francisco, CA 94143-0114, USA*
[b]*University of Berne, Institute of Infectious Diseases, Berne, Switzerland*

Accepted 29 October 2001

Abstract

In the immature brain hydrogen peroxide accumulates after excitotoxic hypoxia–ischemia and is neurotoxic. Immature hippocampal neurons were exposed to *N*-methyl-D-aspartate (NMDA), a glutamate agonist, and hydrogen peroxide (H_2O_2) and the effects of free radical scavenging and transition metal chelation on neurotoxicity were studied. α-Phenyl-*N-tert.*-butylnitrone (PBN), a known superoxide scavenger, attenuated both H_2O_2 and NMDA mediated toxicity. Treatment with desferrioxamine (DFX), an iron chelator, at the time of exposure to H_2O_2 was ineffective, but pretreatment was protective. DFX also protected against NMDA toxicity. TPEN, a metal chelator with higher affinities for a broad spectrum of transition metal ions, also protected against H_2O_2 toxicity but was ineffective against NMDA induced toxicity. These data suggest that during exposure to free radical and glutamate agonists, the presence of iron and other free metal ions contribute to neuronal cell death. In the immature nervous system this neuronal injury can be attenuated by free radical scavengers and metal chelators.

Theme: Disorders of the nervous system

Topic: Ischemia

Keywords: Oxidative stress; Neonate; Reactive oxygen species; Brain injury; Iron

1. Introduction

It is well documented that excitotoxicity and oxidative stress contribute to the pathogenesis of many neurological disorders [13,7]. The immature brain is rich in polyunsaturated fatty acids [9] and is susceptible to peroxidation [6]. In addition, the brain does not have high levels of protective enzymes such as glutathione peroxidase and catalase [6] and thus the generation of free radicals could exceed the level of antioxidant defenses leading to cellular injury [39]. This damage is exacerbated in neonates, where antioxidant defenses are less mature [32] and free iron is more abundant [41]. In a neonatal murine model of hypoxic–ischemic (H–I) injury, pups transgenic for superoxide dismutase 1 (SOD1) had greater brain damage than their nontransgenic littermates [11]. In adult models of ischemia, however, increased SOD1 expression has been associated with neuroprotection [50]. An explanation for this discrepancy is that the effects of SOD in the brain differ during development and maturation [16]. In the adult SOD1 transgenic brain there is a compensatory increase in downstream enzymes like catalase; however no such compensation occurs in the neonatal brain [16]. The imbalance of pro-oxidant and antioxidant defenses leads to the accumulation of H_2O_2.

Immature neurons are particularly susceptible to damage from oxidative stress due to exposure to H_2O_2 [33]. In comparison to more mature neurons in culture, immature neurons experience more cell death at lower concentrations of H_2O_2. This may in part be due to underdeveloped scavenging systems in the setting of a rich iron environ-

*Corresponding author. Tel.: +1-415-502-5820; fax: +1-415-502-5821.

E-mail address: dmf@itsa.ucsf.edu (D.M. Ferriero).

0165-3806/01/$ – see front matter
PII: S0165-3806(01)00302-9

ment. Since the neonatal brain has more free iron than the mature brain [41], the immature brain may be more susceptible to the generation of hydroxyl radicals through the Fenton reaction [49]. The Fenton reaction is a one-electron nonenzymatic transfer reaction in which transition elements generate hydroxyl radicals from H_2O_2. The rate constant for the Fenton reaction is higher for copper than for iron [20] but iron is more abundant. This accessibility coupled with the increased production of intermediates of oxygen reduction such as H_2O_2 generates a prooxidant status in the cell. Thus the neonatal brain may be more susceptible to oxidative stress through this mechanism.

Previous studies have shown that there are many agents that ameliorate neuronal injury by scavenging free radicals after oxidative stress. α-Phenyl-*N-tert.*-butylnitrone (PBN) is a nitrone spin trap that converts free radicals into stable adducts [35]. PBN has been shown to be neuroprotective after focal brain ischemia in vivo [5,52] and is protective after exposure to a variety of excitotoxins in vitro [44,34]. In particular, it has been shown to reduce the levels of hydroxyl radicals after infusion of glutamate into rat striatum [28]. Therefore, a link between glutamate excitotoxicity and free radical generation exists, and this link can be interrupted by free radical scavenging.

Desferrioxamine (DFX) is an iron chelator that could potentially prevent iron involvement in Fenton chemistry [49]. DFX has been shown in some systems to inhibit lipid peroxidation and hydroxyl radical formation thereby decreasing H–I and reperfusion associated brain injury [17,38]. In neonatal mice, DFX has been shown to be protective after focal ischemia and global hypoxia, especially in regions of increased iron stores like the hippocampus [43]. Iron chelators have also been shown to activate a hypoxia stress response through the expression of hypoxia-inducible factor-1 (HIF-1) [47]. In the neonatal brain DFX appears to produce tolerance to H–I, possibly by this mechanism [4].

N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine (-TPEN) is a structural analogue of EDTA which chelates a broad spectrum of heavy metals [2] that could participate in the generation of free radicals during oxidative stress. It has recently been shown that TPEN reduces cortical neuronal cell death in mixed cultures under both normal and acidotic conditions after H_2O_2 [51]. Although this metal chelator has lower specificity for iron, it has a higher cell permeability than DFX.

The purpose of this study is to utilize a primary cell culture of immature neurons to test the relative efficacies of metal chelators and a free radical scavenger in their abilities to rescue hippocampal neurons from oxidative or excitotoxic stress. We report here the protection with a free radical scavenger and metal chelators in immature neurons during both H_2O_2 and NMDA exposure, providing further evidence of the link between excitotoxicity, oxidative stress and neuronal injury in the immature nervous system.

2. Materials and methods

2.1. Primary hippocampal cell culture

Hippocampal cultures were prepared from fetal (E16) CD-1 mice. Cortices were dissected away and the hippocampi were isolated. After the meninges were removed, the hippocampi were placed in a trypsin solution (Sigma, 2 mg/ml in Hank's buffered salt solution) for 10 min at 37°C. This dissociation was stopped by the addition of horse serum and then the cell suspension was centrifuged. The cells were resuspended in astrocyte conditioned media (ACM) supplemented with 10% horse serum. ACM was prepared from media stock (minimum essential media with Earle's salts minus L-glutamine, custom) and supplemented with glucose, glutamine and 10% fetal bovine serum, which had been placed in 75-ml flasks of astrocytes 24 h prior to use. After mechanical trituration, the cell suspensions were diluted in ACM and plated at 1.4×10^6 cells/ml onto 96-well plates (Falcon) that had been coated with poly-D-lysine for optimal cell adhesion. At 30 min after plating, the cells were washed with ACM, reducing the concentration of horse serum to 5%. Astrocyte growth was inhibited by the addition of 10 mM cytosine arabinoside (Ara-C) at day 1 in vitro. The concentration of Ara-C was reduced to 3 mM by a media replacement on days in vitro (div) 2 to ensure an astrocyte population of <5%, confirmed by GFAP staining (Fig. 1). The cultures were maintained in a humidified, 5% CO_2, 37°C incubator. After 6 days in culture, the cells were used for experiments.

Glial cell cultures were utilized for the preparation of ACM using a similar protocol as above but using cortices isolated from postnatal mice (P1-3). These cells were plated in 75-ml flasks and the supernatant used when confluent (~2 weeks in culture).

2.2. Neurotoxicity experiments

Hippocampal cell cultures (div 6) were washed in ACM with only 1% fetal bovine serum and then exposed to neurotoxins (H_2O_2 or NMDA) in the presence or absence of PBN, TPEN, or DFX for 24 h at various doses ranging from 50 μM to 20 mM. Both PBN and TPEN were simultaneously added with H_2O_2 or NMDA, whereas cells were pretreated for 1 h with DFX before adding the neurotoxin. Each condition was run in triplicate, and each experiment was performed at least four times.

2.3. Analysis of cell death

Cell death was quantified by colorimetric assay detecting lactate dehydrogenase (LDH) activity released into the supernatant 24 h after treatment exposure. Cell death was confirmed by trypan blue inclusion. Each experiment was

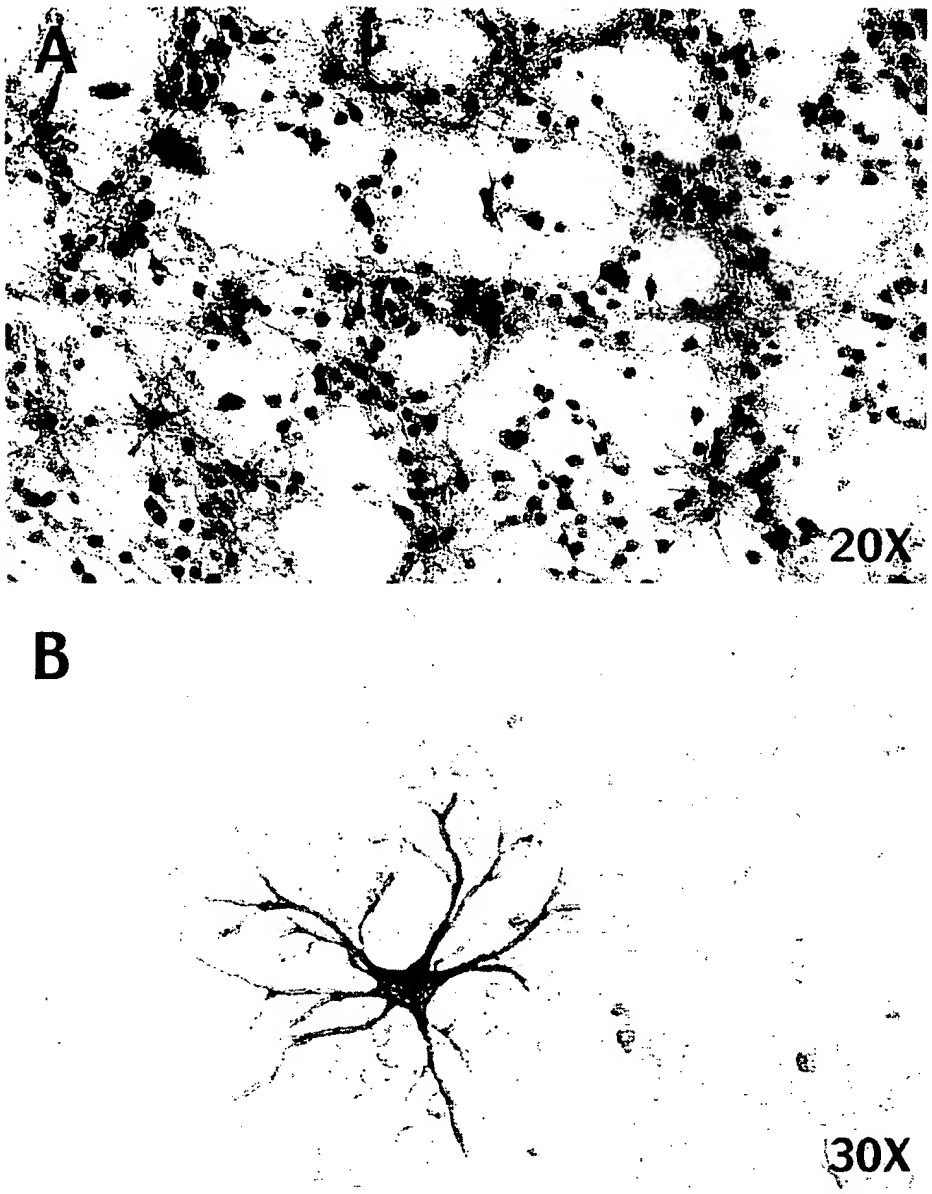


Fig. 1. Primary neuronal cultures at div 6. (A) Neuron specific enolase (NSE) immunostaining reveals healthy neurons. (B) Glial fibrillary acidic protein (GFAP) immunostaining reveals little astrocyte contamination.

normalized by subtracting the background levels of LDH produced from the 'no-treatment' wells [24].

2.4. Statistical analysis

All data are represented as mean±S.E.M. Either Student's *t*-test for paired comparisons or ANOVA for comparisons among groups with posthoc testing were performed where appropriate.

3. Results

3.1. PBN attenuates cell death

The potential of a free radical scavenger in attenuating

cell death was examined. Previously the susceptibility of immature neurons to exposure to H_2O_2 in vitro had been shown [33]. PBN showed a dose dependent neuroprotection in the immature hippocampal cultures exposed to sublethal (50 μM) concentrations of H_2O_2 (Fig. 2). At the highest concentrations of PBN used (10 mM), cell death was reduced fourfold. Concentrations of PBN in this range have been reported in other cell culture systems [36] and are consistent with those used in vivo. PBN was also protective against exposure to lethal (100 μM) concentrations of H_2O_2 but in a less dose dependent fashion. Additionally, PBN attenuated excitotoxic death due to 500 μM NMDA exposure in these immature cultures, but only at high concentration (10 mM) (Fig. 3).

3.2. Neuroprotective effect of DFX requires pretreatment

It was next investigated whether free metal ions play a role in H_2O_2 induced toxicity by catalyzing injurious hydroxyl radical formation. In initial experiments, we examined the effect of DFX on damage produced by H_2O_2 in hippocampal neuronal cultures. Treatment with DFX at the time of exposure to H_2O_2 was ineffective due to limited cell penetration. Cells were pretreated with DFX for 1 h prior to H_2O_2 exposure. When exposed to sublethal H_2O_2 (50 μM), a dose dependent reduction in cell death was seen with doses ranging from 500 μM to 20 mM. Pretreatment reduced cell death by 70% when exposed to a sublethal concentration of H_2O_2 (50 μM) and 80% with a lethal concentration of H_2O_2 (100 μM) (Fig. 4). DFX was less effective in protecting the neurons from NMDA induced cell death. The highest dose of DFX tested (20 mM) reduced cell death from 70% to ~35% (Fig. 5).

3.3. TPEN is neuroprotective against H_2O_2 induced neurotoxicity but not NMDA neurotoxicity

To test whether this limited efficacy of DFX could be due to hydroxyl radical formation by metals not chelated effectively by DFX, the effect of TPEN, a metal chelator with high affinities for a broad spectrum of transition metal ions including zinc, iron and copper was examined. 10 μM TPEN rescued over 70% of the neurons during both lethal and sublethal exposures to H_2O_2 — cell death was reduced from 100% to approximately 28% at lethal exposures and reduced from 66 to 12% at sublethal exposures (Fig. 6). TPEN was ineffective at protecting neurons from NMDA induced neurotoxicity at concentrations that were maximally protective against toxicity from H_2O_2 (Fig. 3).

4. Discussion

The present experiments have established that both metal chelators and a free radical scavenger are capable of rescuing immature neurons from H_2O_2 and NMDA in-

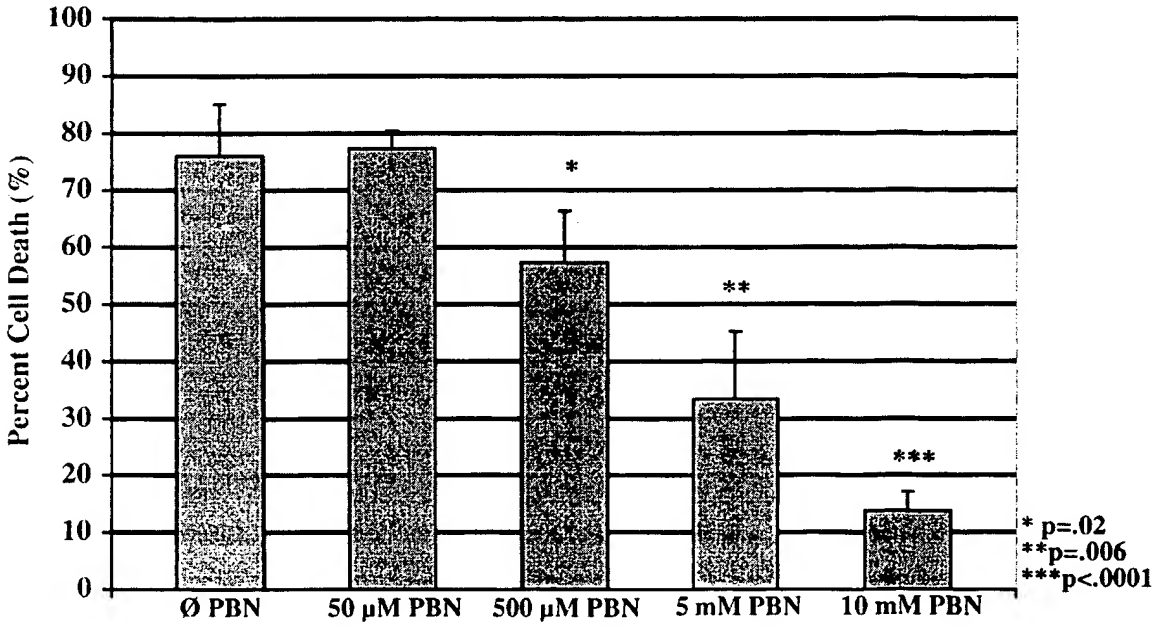


Fig. 2. PBN exhibits a neuroprotective dose response in div 6 cultures treated 50 μM H_2O_2 for 24 h. Values shown are percentages (mean±S.E.M.) normalised to unexposed untreated control hippocampal neurons. Each bar represents values from at least three experiments; $P=0.02$ for 50 μM PBN; $P=0.006$ for 5 mM PBN; $P=0.0001$ for 10 mM PBN (ANOVA).

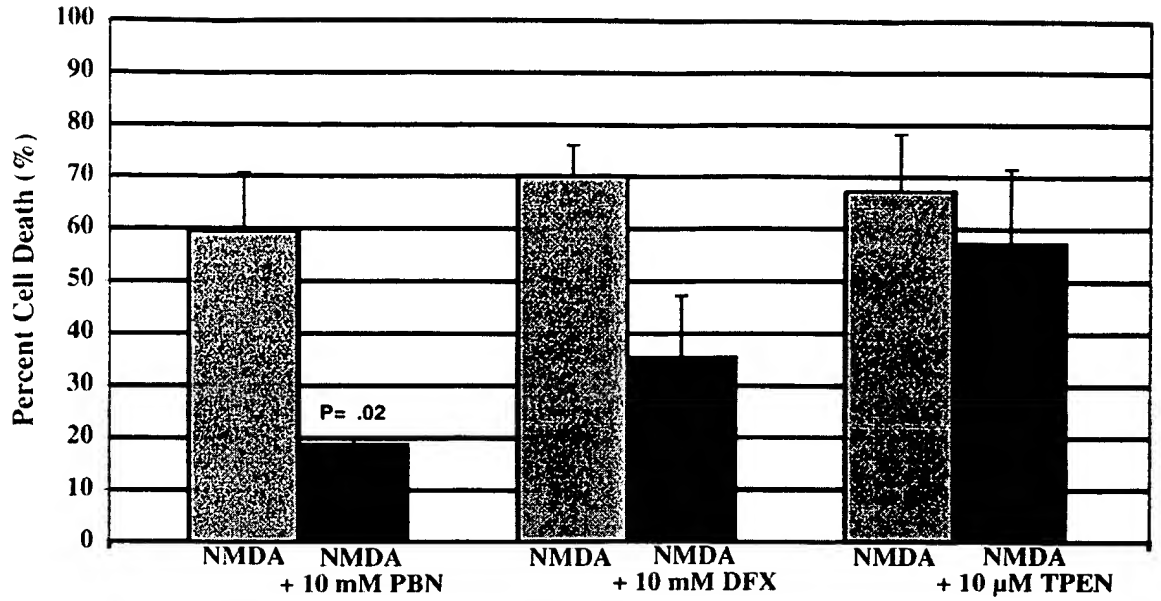


Fig. 3. PBN is more effective than DFX in protecting primary hippocampal neurones at div 6 from NMDA induced cell death. $P=0.02$ (paired t-test). TPN is ineffective at concentrations that protect cells from H_2O_2. Values shown are percentages (mean±S.E.M.) normalised to unexposed untreated control hippocampal neurons. Each bar represents values from at least three experiments.

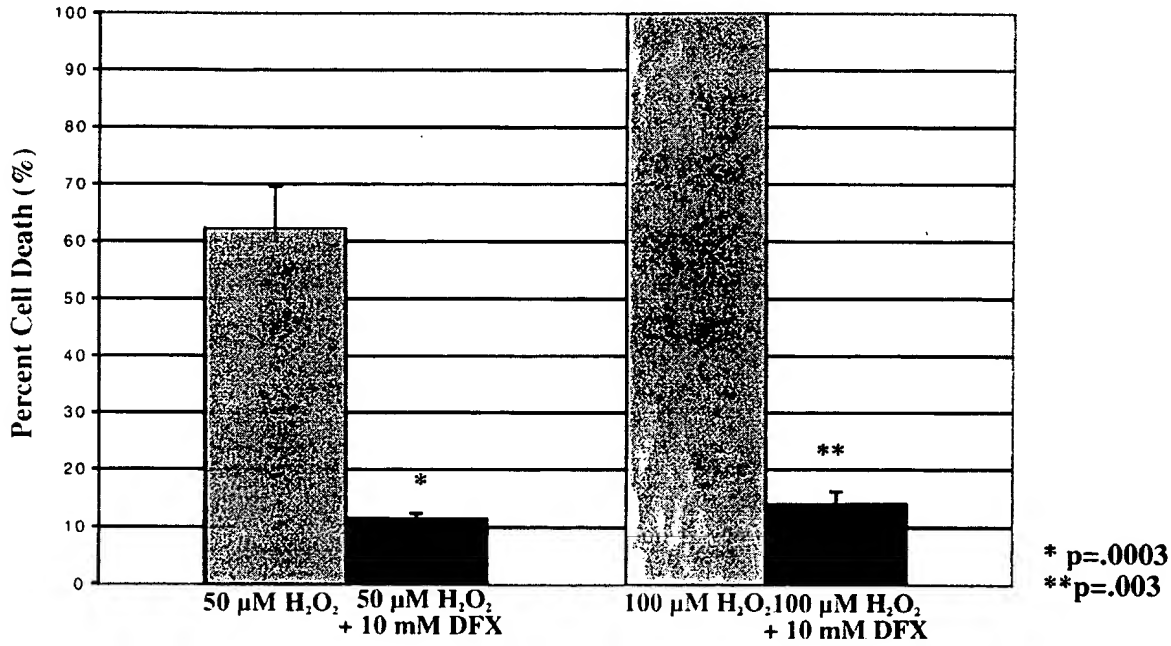


Fig. 4. DFX reduces cell death induced by 2-h exposure to both sublethal and lethal concentrations of H_2O_2 in div 6 cultures. Values shown are percentages (mean±S.E.M.) normalised to unexposed untreated control hippocampal neurons. Each bar represents values from at least three experiments; $P=0.0003$ for 10 mM DFX with sublethal (50 μM) dose H_2O_2 and $P=0.003$ for 10 mM DFX with lethal (100 μM) dose H_2O_2; (paired t-test).

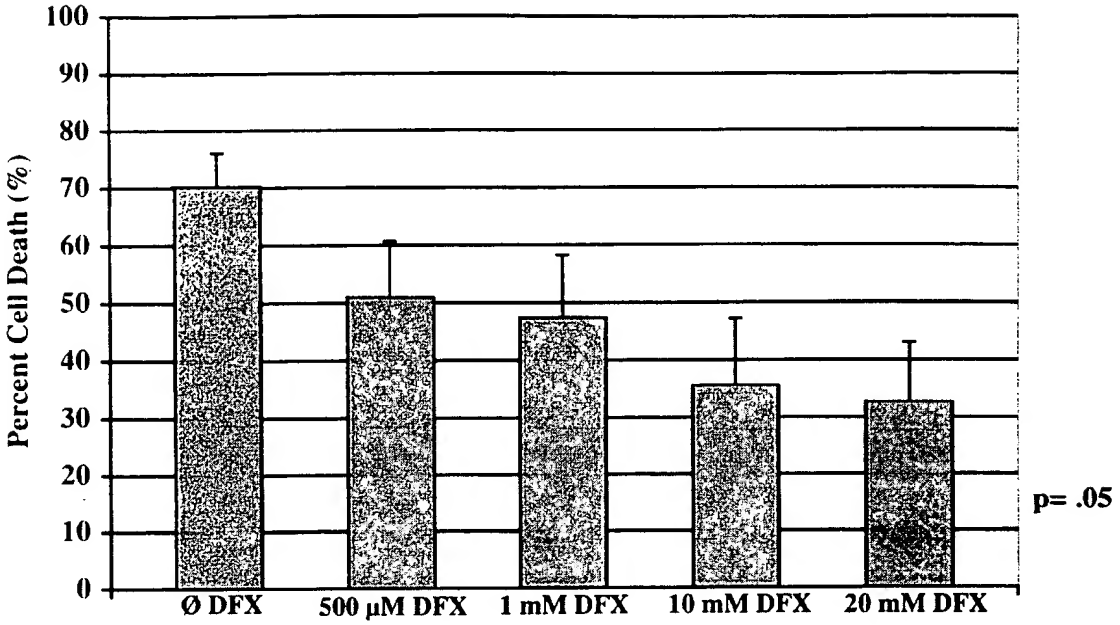


Fig. 5. DFX reduces cell death induced by exposure to 500 µM NMDA in div 6 cultures in a dose dependent manner. Values shown are percentages (mean±S.E.M.) normalised to unexposed untreated control hippocampal neurons. Each bar represents values from at least three experiments; $P=0.05$ (ANOVA).

duced injury. This neuroprotection from different types of toxins suggests multiple pathways between excitotoxic (NMDA) and oxidative (H_2O_2) stress in the immature nervous system. Both PBN and DFX can protect against both types of injury, while the chelator TPEN is only effective against toxicity from H_2O_2. Our data support this hypothesis that there are multiple pathways of free radical production since the broader spectrum scavenger, PBN,

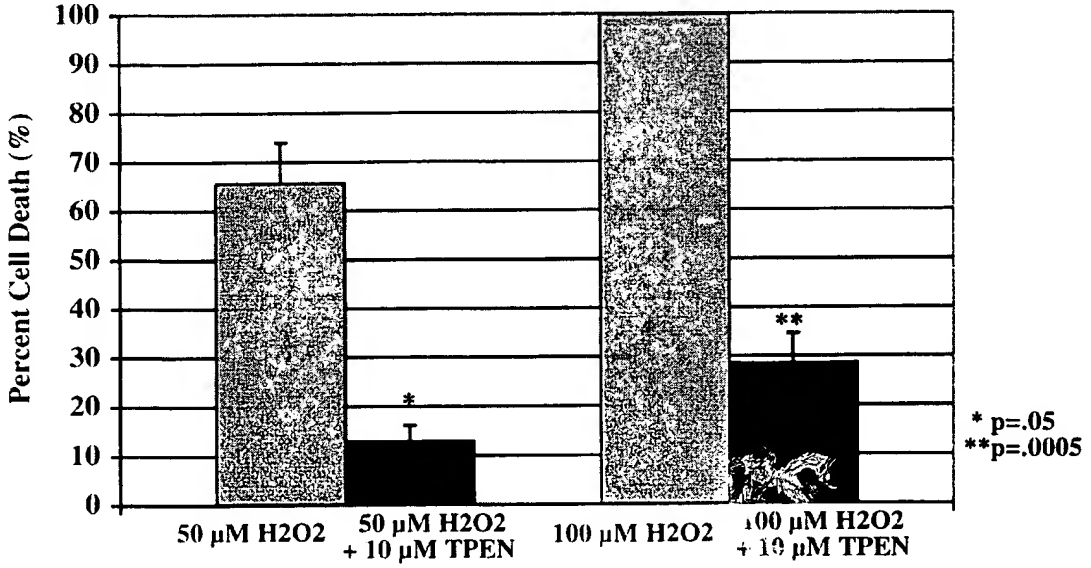


Fig. 6. TPEN significantly reduces cell death induced by 24-h exposure to both sublethal and lethal concentrations of H_2O_2. Values shown are percentages (mean±S.E.M.) normalised to unexposed untreated control hippocampal neurons. Each bar represents values from at least three experiments; $P=0.05$ for 50 µM H_2O_2 and 10 µM TPEN; $P=0.0005$ for 100 µM H_2O_2 and 10 µM TPEN (paired t-test).

was more effective against NMDA induced injury than TPEN or DFX, suggesting that chelation of free radicals is not the only mechanism responsible for neuroprotection. The compounds tested did not equally prevent NMDA induced toxicity. Although there is mitochondrial production of reactive oxygen species in cortical neurons following exposure to NMDA [14], increased intracellular calcium caused by NMDA receptor activation can potentiate cell death by other pathways, including disruption of the mitochondrial membrane potential [31]. Lafon-Cazal et al. [27] found that NMDA induces superoxide formation through an increase in intracellular calcium and the release of arachidonic acid that in turn produces more free radicals.

Despite the differences in NMDA and H_2O_2 toxicity, iron or other transition metals may be implicated in this injury pathway. H–I to the developing brain increases free metal ions [19,37] which then can participate in the Fenton reaction. Iron accumulation occurs very early in the immature brain after injury, as early as 4 h after the insult [37], whereas in the mature nervous system changes are not seen until 4–8 weeks after an ischemic insult [26]. This early appearance of iron is seen in reactive glia and follows the course of cortical blood vessels. In particular, the CA1 region of the hippocampus exhibits accumulation of iron, and in studies in neonatal mouse brain after H–I, there is marked accumulation of iron reaction product throughout the damaged hippocampus [45]. In support of the role of iron in the pathophysiology of neuronal damage in vivo are studies showing neuroprotection with DFX in both neonatal rats [38] and mice [43]. In the former study, DFX was shown to accumulate in the brain at concentrations in the range of 100–200 μM. Iron has been shown to potentiate other forms of cell death in the developing nervous system as well. Iron supplementation aggravates periventricular cystic white matter lesions in newborn mice caused by ibotenate intracerebrovascular injections [12] and disrupts maze learning and motor activity in adult mice exposed to iron during restricted postnatal periods [15].

4.1. PBN efficacy

PBN may be particularly effective in protecting hippocampal neurons because of its lipophilicity (it can penetrate the blood–brain barrier) and its ability to scavenge free radicals. This molecule scavenges hydroxyl radicals in particular [28], which may be generated intracellularly [5]. PBN reduces the formation of 2,3-DHBA in rat striatum exposed to 500 μM glutamate while reducing the volume of the infarction by 19% [28], data providing an additional link between glutamate toxicity and free radical formation. Another study investigating the toxicity of malonate and 3-acetylpyridine in adult rat striatum showed that PBN reduced hydroxyl radical formation by the same method, while having no effect on lactate production or energy preservation [44]. Perhaps the most direct link is provided by the fact that NMDA exposure leads to superoxide generation in cultures of cerebellar neurons [27].

4.2. DFX efficacy

Though DFX has been shown to attenuate H_2O_2 induced neuronal death in mixed cortical cultures [51], it penetrates cells very slowly [18]. This was evident in the protective effect of pretreatment with DFX but not cotreatment. DFX specifically chelates iron and the formation constant of the DFX–Fe (III) complex is $\approx 10^{31}$, which is significantly higher than the formation constants of DFX–Cu^{2+} (10^{14}) and DFX–Zn^{2+} (10^{11}) [18]. Ahn et al. [1] and Aruoma et al. [3] found copper to be more damaging than iron during H_2O_2 exposure, though zinc has also been implicated in neuronal injury [23]. At millimolar concentrations of DFX, the compound can act as a scavenger of superoxide and hydroxical radicals, much like PBN [21,42]. DFX is known to upregulate hypoxia inducible factor-1 (HIF1) [48]. In rat cortical neuronal cultures, DFX protected cells from oxidative stress produced by homocysteic acid, a glutamate analog. Nuclear extracts from DFX treated cells showed HIF1 induction, with supershift analysis confirming that the induced complexes were composed of HIF 1 and ATF1/CREB at concentrations as low as 10 μM DFX [53]. In neonatal rats, DFX pretreatment induces HIF1 and protects the brain after a H–I insult [4]. The mechanism of neuroprotection by DFX is currently being investigated.

4.3. TPEN efficacy

TPEN is also lipophilic, but chelates other transition metal ions, such as copper and zinc. These other ions could be involved in Fenton reactions that produce the injurious hydroxyl radical, or could be toxic through other mechanisms [22,23,8]. TPEN preferentially chelates zinc [1,30] so our result showing neuroprotection from TPEN at very low concentrations may implicate the involvement of zinc in H_2O_2 toxicity. Chelatable zinc has been found in degenerating neuronal perikarya after ischemia [25] and thus the high permeability of TPEN may be required to chelate intracellular release of endogenous zinc [10]. Additionally, TPEN has been shown to provide continued action of zinc chelation [29], which may explain its efficacy at low doses. TPEN was ineffective against NMDA at concentrations that are protective against H_2O_2 toxicity, suggesting that chelation is not the only mechanism responsible for neuroprotection. Zinc has been shown to block NMDA mediated neuroexcitation [40], thus TPEN may be chelating the zinc that is attenuating cell death.

In summary, it appears that transition metals play an important role in the genesis of injury to the immature hippocampal neuron. The data presented here support the

role of excitotoxic and oxidative stress as a major component of toxicity to the immature neuron. The potential benefit of directed therapy against oxidative stress in the form of free radical scavengers and metal chelators as demonstrated here raises the possibility of therapeutic trials in the human asphyxiated newborn brain. With this mounting evidence pointing to the unique susceptibility of the immature brain to oxidative stress [16,43,46], more appropriate therapeutics can be designed to lessen the damage caused by these devastating events.

Acknowledgements

The authors would like to thank Carla Francisco for expert technical assistance. Work supported by NS 35902 to Drs. Donna M. Ferriero and Martin G. Täuber.

References

[1] Y.H. Ahn, Y.H. Kim, S.H. Hong, J.Y. Koh, Depletion of intracellular zinc induces protein synthesis-dependent neuronal apoptosis in mouse cortical culture, Exp. Neurol. 154 (1998) 47–56, http://www.idealibrary.com/links/citation/0014-4886/154/47 http://www.idealibrary.com/links/toc/exnr/.

[2] P. Arslan, F. Di Virgilio, M. Beltrame, R.Y. Tsien, T. Pozzan, Cytosolic Ca^{2+} homeostasis in Ehrlich and Yoshida carcinomas. A new, membrane-permeant chelator of heavy metals reveals that these ascites tumor cell lines have normal cytosolic free Ca^{2+}, J. Biol. Chem. 260 (1985) 2719–2727.

[3] O.I. Aruoma, B. Halliwell, E. Gajewski, M. Dizdaroglu, Copper-ion-dependent damage to the bases in DNA in the presence of hydrogen peroxide, Biochem. J. 273 (1991) 601–604.

[4] M. Bergeron, J.M. Gidday, A.Y. Yu, G.L. Semenza, D.M. Ferriero, F.R. Sharp, Role of hypoxia-inducible factor-1 (HIF-1) in hypoxia-induced ischemic tolerance in neonatal rat brain, Ann. Neurol. 48 (2000) 285–296.

[5] X. Cao, J.W. Phillis, α-Phenyl-*tert*.-butyl-nitrone reduces cortical infarct and edema in rats subjected to focal ischemia, Brain Res. 644 (1994) 267–272.

[6] J.M. Carney, R.A. Floyd, Protection against oxidative damage to CNS by α-phenyl-*tert*.-butylnitrone (PBN) and other spin-trapping agents: a novel series of nonlipid free radical scavengers, J. Mol Neurosci. 3 (1991) 47–57.

[7] P.H. Chan, L. Chu, S.F. Chen, E.J. Carlson, C.J. Epstein, Reduced neurotoxicity in transgenic mice overexpressing human copper–zinc-superoxide dismutase, Stroke 21 (1990) III80–III82.

[8] Q.X. Chen, K.L. Perkins, R.K.S. Wong, Zn^{2+} blocks the NMDA- and Ca^{2+}-triggered postexposure current Ipe in hippocampal pyramidal cells, J. Neurophysiol. 79 (1998) 1124–1126, http://jn.physiology.org/cgi/content/full/79/2/1124 http://jn.physiology.org.

[9] J.T. Coyle, P. Puttfarcken, Oxidative stress, glutamate and neurodegenerative disorders, Science 262 (1993) 689–695.

[10] M.P. Cuajungco, G.J. Lees, Diverse effects of metal chelating agents on the neuronal cytotoxicity of zinc in the hippocampus, Brain Res. 799 (1998) 97–107, http://www.sciencedirect.com/science?*ob=GatewayURLand*origin=CDLand*urlversion=3and*method=citationSearch&*volkey=00068993%23799%2397&*version=1&md5=c9c487c3913c4f06d8b8185bfaef7633 http://www.sciencedirect.com/science/journal/00068993.

[11] J.S. Ditelberg, R.A. Sheldon, C.J. Epstein, D.M. Ferriero, Brain injury after perinatal hypoxia–ischemia is exacerbated in copper/zinc superoxide dismutase transgenic mice, Pediatr. Res. 39 (1996) 204–208.

[12] M.A. Dommergues, J. Gallego, P. Evrard, P. Gressens, Iron supplementation aggravates periventricular cystic white matter lesions in newborn mice, Eur. J. Paediatr. Neurol. 2 (1998) 313–318.

[13] L.L. Dugan, D.W. Choi, Excitotoxicity, free radicals and cell membrane changes, Ann. Neurol. 35 (1994) S17–21.

[14] L.L. Dugan, S.L. Sensi, L.M. Canzoniero, S.D. Handran, S.M. Rothman, T.S. Lin, M.P. Goldberg, D.W. Choi, Mitochondrial production of reactive oxygen species in cortical neurons following exposure to *N*-methyl-D-aspartate, J. Neurosci. 15 (1995) 6377–6388.

[15] A. Fredriksson, N. Schröder, P. Eriksson, I. Izquierdo, T. Archer, Maze learning and motor activity deficits in adult mice induced by iron exposure during a critical postnatal period, Brain Res. Dev. Brain Res. 119 (2000) 65–74, http://www.sciencedirect.com/science?*ob=GatewayURL&*origin=CDL&*urlversion=3&*method=citationSearch&*volkey=01653806%23119%2365&*version=1&md5=e81575a411f7c338604c2fa4cfda9f0d http://www.sciencedirect.com/science/journal/01653806.

[16] H.J. Fullerton, J.S. Ditelberg, S.F. Chen, D.P. Sarco, P.H. Chan, C.J. Epstein, D.M. Ferriero, Copper/zinc superoxide dismutase transgenic brain accumulates hydrogen peroxide after perinatal hypoxia–ischemia, Ann. Neurol. 44 (1998) 357–364.

[17] J.M. Gutteridge, R. Richmond, B. Halliwell, Inhibition of the iron-catalysed formation of hydroxyl radicals from superoxide and of lipid peroxidation by desferrioxamine, Biochem. J. 184 (1979) 469–472.

[18] B. Halliwell, Protection against tissue damage in vivo by desferrioxamine: what is its mechanism of action?, Free Radic. Biol. Med. 7 (1989) 645–651.

[19] B. Halliwell, Reactive oxygen species and the central nervous system, J. Neurochem. 59 (1992) 1609–1623.

[20] B. Halliwell, J.M.C. Gutteridge, Free Radicals in Biology and Medicine, 2nd Edition, Clarendon Press; Oxford University Press, New York; Oxford, 1989.

[21] S. Hoe, D.A. Rowley, B. Halliwell, Reactions of ferrioxamine and desferrioxamine with the hydroxyl radical, Chem. Biol. Interact. 41 (1982) 75–81.

[22] L. Jornot, H. Petersen, A.F. Junod, Hydrogen peroxide-induced DNA damage is independent of nuclear calcium but dependent on redox-active ions, Biochem. J. 335 (1998) 85–94.

[23] Y.H. Kim, E.Y. Kim, B.J. Gwag, S. Sohn, J.Y. Koh, Zinc-induced cortical neuronal death with features of apoptosis and necrosis: mediation by free radicals, Neuroscience 89 (1999) 175–182, http://www.sciencedirect.com/science?*ob=GatewayURL&*origin=CDL&*urlversion=3&*method=citationSearch&*volkey=03064522%2389%23175&*version=1&md5=962d61d20450d4879dd654e2443143e3 http://www.sciencedirect.com/science/journal/03064522.

[24] J.Y. Koh, D.W. Choi, Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay, J. Neurosci. Methods 20 (1987) 83–90.

[25] J.Y. Koh, S.W. Suh, B.J. Gwag, Y.Y. He, C.Y. Hsu, D.W. Choi, The role of zinc in selective neuronal death after transient global cerebral ischemia, Science 272 (1996) 1013–1016, http://www.sciencemag.org/cgi/content/full/272/5264/1013 http://www.sciencemag.org.

[26] Y. Kondo, N. Ogawa, M. Asanuma, Z. Ota, A. Mori, Regional differences in late-onset iron deposition, ferritin, transferrin, astrocyte proliferation and microglial activation after transient forebrain ischemia in rat brain, J. Cereb. Blood Flow Metab. 15 (1995) 216–226.

[27] M. Lafon-Cazal, S. Pietri, M. Culcasi, J. Bockaert, NMDA-depen-

dent superoxide production and neurotoxicity, Nature 364 (1993) 535–537.

[28] E. Lancelot, M.L. Revaud, R.G. Boulu, M. Plotkine, J. Callebert, Alpha-phenyl-*N*-*tert*.-butylnitrone attenuates excitotoxicity in rat striatum by preventing hydroxyl radical accumulation, Free Radic. Biol. Med. 23 (1997) 1031–1034, http://www.sciencedirect.com/science?*ob=GatewayURL&*origin=CDL&*urlversion=3&*method=citationSearch&*volkey=08915849%2323%231031&*version=1&md5=82736b0339a3ff91041c875f98c4cf2d http://www.sciencedirect.com/science/journal/08915849.

[29] G.J. Lees, M.P. Cuajungco, W. Leong, Effect of metal chelating agents on the direct and seizure-related neuronal death induced by zinc and kainic acid, Brain Res. 799 (1998) 108–117, http://www.sciencedirect.com/science?*ob=GatewayURL&*origin=CDL&*urlversion=3&*method=citationSearch&*volkey=00068993%23799%23108&*version=1&md5=2f6f5a77da076cbc560664c32c6fd011 http://www.sciencedirect.com/science/journal/00068993.

[30] M. Marini, D. Musiani, Micromolar zinc affects endonucleolytic activity in hydrogen peroxide-mediated apoptosis, Exp. Cell Res. 239 (1998) 393–398, http://www.idealibrary.com/links/citation/0014-4827/239/393 http://www.idealibrary.com/links/toc/excr/.

[31] M.P. Mattson, Y. Zhang, S. Bose, Growth factors prevent mitochondrial dysfunction, loss of calcium homeostasis and cell injury, but not ATP depletion in hippocampal neurons deprived of glucose, Exp. Neurol. 121 (1993) 1–13.

[32] I. Mavelli, A. Rigo, R. Federico, M.R. Ciriolo, G. Rotilio, Superoxide dismutase, glutathione peroxidase and catalase in developing rat brain, Biochem. J. 204 (1982) 535–540.

[33] R.E. Mischel, Y.S. Kim, R.A. Sheldon, D.M. Ferriero, Hydrogen peroxide is selectively toxic to immature murine neurons in vitro, Neurosci. Lett. 231 (1997) 17–20.

[34] N. Nakao, E.M. Grasbon-Frodl, H. Widner, P. Brundin, Antioxidant treatment protects striatal neurons against excitotoxic insults, Neuroscience 73 (1996) 185–200.

[35] C.N. Oliver, P.E. Starke-Reed, E.R. Stadtman, G.J. Liu, J.M. Carney, R.A. Floyd, Oxidative damage to brain proteins, loss of glutamine synthetase activity and production of free radicals during ischemia–reperfusion-induced injury to gerbil brain, Proc. Natl. Acad. Sci. USA 87 (1990) 5144–5147.

[36] C. Olsen, A. Rustad, F. Fonnum, R.E. Paulsen, B. Hassel, 3-Nitropropionic acid: an astrocyte-sparing neurotoxin in vitro, Brain Res. 850 (1999) 144–149, http://www.sciencedirect.com/science?*ob=GatewayURL&*origin=CDL&*urlversion=3&*method=citationSearch&*volkey=00068993%23850%23144&*version=1&md5=c41a04b873a3502b7a743d7b82052d12.

[37] C. Palmer, S.L. Menzies, R.L. Roberts, G. Pavlick, J.R. Connor, Changes in iron histochemistry after hypoxic–ischemic brain injury in the neonatal rat, J. Neurosci. Res. 56 (1999) 60–71, http://mddb.wiley.com/db/mdresolve.cgi?issn=0360-4012&volume=56&issue=1&first*page=60 http://www.interscience.wiley.com/jpages/0360-4012.

[38] C. Palmer, R.L. Roberts, C. Bero, Deferoxamine posttreatment reduces ischemic brain injury in neonatal rats, Stroke 25 (1994) 1039–1045.

[39] M.C. Papadopoulos, I.L. Koumenis, T.Y. Yuan, R.G. Giffard, Increasing vulnerability of astrocytes to oxidative injury with age despite constant antioxidant defenses, Neuroscience 82 (1998) 915–925, http://www.sciencedirect.com/science?*ob=GatewayURL&*origin=CDL&*urlversion=3&*method=citationSearch&*volkey=03064522%2382%23915&*version=1&md5=e5914e3d2571de5a152d6f5e663b70c3 http://www.sciencedirect.com/science/journal/03064522.

[40] S. Peters, J. Koh, D.W. Choi, Zinc selectively blocks the action of *N*-methyl-D-aspartate on cortical neurons, Science 236 (1987) 589–593, http://links.jstor.org/sici?sici=0036-8075%2819870501%29236%3A4801%3C589%3AZSBTAO%3E2.0.CO%3B2-E http://www.jstor.org/journals/00368075.html.

[41] A.J. Roskams, J.R. Connor, Iron, transferrin and ferritin in the rat brain during development and aging, J. Neurochem. 63 (1994) 709–716.

[42] D. Sabourault, C. Ribiere, R. Nordmann, C. Houee-Levin, C. Ferradini, Gamma and pulse radiolysis investigation of the reaction of desferrioxamine with superoxide anions [published erratum appears in Int. J. Radiat. Biol. 1990;57(2):448], Intern. J. Radiat. Biol. 56 (1989) 911–921.

[43] D. Sarco, J. Becker, C. Palmer, R.A. Sheldon, D.M. Ferriero, The neuroprotective effect of deferoxamine in the hypoxic–ischemic immature mouse brain, Neurosci. Lett. 282 (2000) 113–116.

[44] J.B. Schulz, D.R. Henshaw, D. Siwek, B.G. Jenkins, R.J. Ferrante, P.B. Cipolloni, N.W. Kowall, B.R. Rosen, M.F. Beal, Involvement of free radicals in excitotoxicity in vivo, J. Neurochem. 64 (1995) 2239–2247.

[45] R.A. Sheldon, J. Hall, L. Noble, D.M. Ferriero, Hippocampal neuronal death following hypoxic–ischemic injury in neonatal mice, Soc. Neurosci. Abstr. 24 (1998) 1955.

[46] Z.S. Vexler, D.M. Ferriero, Molecular and biochemical mechanisms of perinatal brain injury, Semin. Neonatol. 6 (2001) 99–108.

[47] G.L. Wang, G.L. Semenza, Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia, J. Biol. Chem. 268 (1993) 21513–21518.

[48] G.L. Wang, G.L. Semenza, Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction, Blood 82 (1993) 3610–3615.

[49] C.C. Winterbourn, Toxicity of iron and hydrogen peroxide: the Fenton reaction, Toxicol. Lett. 82–83 (1995) 969–974.

[50] G. Yang, P.H. Chan, J. Chen, E. Carlson, S.F. Chen, P. Weinstein, C.J. Epstein, H. Kamii, Human copper–zinc superoxide dismutase transgenic mice are highly resistant to reperfusion injury after focal cerebral ischemia, Stroke 25 (1994) 165–170.

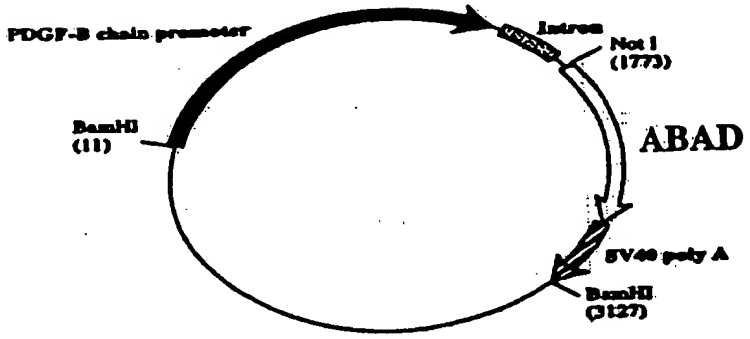
[51] W. Ying, S.K. Han, J.W. Miller, R.A. Swanson, Acidosis potentiates oxidative neuronal death by multiple mechanisms, J. Neurochem. 73 (1999) 1549–1556.

[52] T.L. Yue, J.L. Gu, P.G. Lysko, H.Y. Cheng, F.C. Barone, G. Feuerstein, Neuroprotective effects of phenyl-*t*-butyl-nitrone in gerbil global brain ischemia and in cultured rat cerebellar neurons, Brain Res. 574 (1992) 193–197.

[53] K. Zaman, H. Ryu, D. Hall, K. O'Donovan, K.I. Lin, M.P. Miller, J.C. Marquis, J.M. Baraban, G.L. Semenza, R.R. Ratan, Protection from oxidative stress-induced apoptosis in cortical neuronal cultures by iron chelators is associated with enhanced DNA binding of hypoxia-inducible factor-1 and ATF-1/CREB and increased expression of glycolytic enzymes, p21(waf1/cip1), and erythropoietin, J. Neurosci. 19 (1999) 9821–9830, http://www.jneurosci.org/cgi/content/full/19/22/9821 http://www.jneurosci.org.

Figure 1

1A: PD-huABAD construct (5.8)



1B:

PD-huABAD transgenic cassette (3.1 kb)